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Neuroplasticity of the Dendritic Spine

Early Dysfunction in Amyotrophic Lateral Sclerosis

by

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Located in Chapter 1

Candidate was first author. Authors 7 and 8 contributed to the idea, its formalisation and development. Authors 3 and 4 contributed to the electrophysiological experiments. Authors 2, 5 and 4 contributed to experimental protocols and development. Author 6 contributed to obtaining the TDP-43^{A315T} mouse model.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

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STATEMENT OF ETHICAL CONDUCT

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

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4. **Handley E,** Pitman K, Dawkins E, Young K, Jiang T, Turner B, Dickson T and Blizzard C. Synaptic dysfunction of layer V pyramidal neurons precedes neurodegeneration in a mouse model of TDP-43 proteinopathies. *Presented at the 36th Annual Australian Neuroscience Society Meeting, Hobart, Australia 2016*

5. **Handley E,** Pitman K, Dawkins E, Young K, Jiang T, Turner B, Dickson T and Blizzard C. Synaptic dysfunction of layer V pyramidal neurons precedes neurodegeneration in a mouse

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6. **Handley E,** Dawkins E, Clark R, Turner B, Dickson T and Blizzard C. Spine loss is an early pathogenic event in the TDP-43^{A315T} mouse model of Amyotrophic Lateral Sclerosis. *Presented at the 26th International Symposium on ALS/MND, Orlando, USA 2015*

7. **Handley E**, Dawkins E, Clark R, Turner B, Dickson T and Blizzard C. Spine loss is an early pathogenic event in the TDP-43^{A315T} mouse model of Amyotrophic Lateral Sclerosis. *Presented at the MND Australia Annual Research Meeting, Sydney, Australia 2015*

8. **Handley E,** Dawkins E, Clark R, Turner B, Dickson T and Blizzard C. Spine loss is an early pathogenic event in the TDP-43^{A315T} mouse model of Amyotrophic Lateral Sclerosis. *Presented at the inaugural Asia Pacific FTD/MND Meeting, Sydney, Australia 2015.* ***Winner of the best poster presentation.***

9. **Handley E,** Turner B, Dickson T and Blizzard C. Dendritic spine alterations in the TDP-43^{A315T} mouse model of Amyotrophic Lateral Sclerosis. *Presented at the MND Australia Annual Research Meeting, Melbourne, Australia 2014.* ***Shortlisted for the student poster prize.***

SUMMARY

Neurodegenerative diseases are heterogeneous disorders that share common features of progression, whereby vulnerable cortical regions and neuronal populations are susceptible to disease insults during a prolonged preclinical period. At symptom onset, it is theorised that potential compensatory mechanisms mediating these disease insults fail, and overt symptoms then arise. Synaptic dysfunction is one such pathological pathway that has been repeatedly identified in a range of neurodegenerative diseases, yet the initiating mechanism resulting in the dysregulation of synaptic connections has not been identified. Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease, and features the select vulnerability of the motor system and degeneration of motor neurons, for which we have no effective cures or treatments. Critical insight as to which processes may underlie disease onset and progression have come from studies identifying early changes to the excitability of the motor cortex of ALS patients, and increasingly the dysfunction of the synapse has been linked to these network changes. Thus there is an increased interest in the potential for early dysregulation of the synaptic compartment to initiate pathological disease pathways during preclinical periods. The primary component of characteristic disease aggregates in ALS patient tissue is the DNA/RNA-binding protein TDP-43; in disease states, the protein mislocalises to the nucleus and is sequestered into toxic inclusions within the cytoplasm. Though previous research has focused on the role of TDP-43 within the nuclear and cytoplasmic compartments, increasing evidence indicates the protein has normal roles more distally- particularly at the dendritic spine. The dendritic spine is the primary postsynaptic compartment of glutamatergic neurons and is essential for the regulation of neuroplasticity. Therefore, the aim of this thesis was to investigate how and when misprocessing of TDP-43 may impact the postsynaptic compartment at the dendritic spine, and how this may then impact neuroplasticity. Further, the fundamental features of the motor cortex were examined in order to better understand what may render the region vulnerable to dysfunction mediated by changes at the dendritic spine.

The current thesis investigated the potential for misprocessed TDP-43 to mediate dendritic spine dysfunction by first establishing a timeline of synaptic alterations in the motor cortex of the *TDP-43^{A315T}* mouse model of ALS. The aim of this first study was to investigate whether the synapse is dysfunctional over a disease time course, in order to identify the earliest occurring disease events mediated by TDP-43 using immunohistochemical techniques. Subsequently, neuroplasticity of the dendritic spine was investigated within the motor cortex under normal conditions using cranial window surgeries in conjunction with 2-photon laser scanning microscopy (2PLSM), in order to identify features that may potentially underlie the vulnerability of the region to disease-linked pathology in real time. An additional aim of this second study was to investigate the influence of the sex hormone

oestrogen and age on dendritic spine populations within discrete cortical regions, to better understand factors that may influence the incidence of disease within populations. The final study investigated real-time changes at the dendritic spine in the presence of the *TDP-43^{A315T}* mutation, within male and female mice having undergone cranial window surgeries and 2PLSM.

This thesis determined that synaptic dysfunction is a presymptomatic disease event in the *TDP-43^{A315T}* mouse, occurring specifically in the motor cortex. Dendritic spine density was significantly reduced prior to overt symptoms and cell loss, occuring concurrently with a hypoexcitable phenotype. These changes were targeted within layer V pyramidal neurons of the motor cortex at P60, prior to dendritic spine density changes being evident by P90 within both the motor and somatosensory cortices. These findings highlight the select vulnerability of the motor cortex to TDP-43-mediated dysfunction at the dendritic spine, and thus the features that render this region susceptible to disease were next investigated. This was explored investigating real-time changes in dendritic spines within the motor and somatosensory cortices of *Thy1-YFP* mice, to establish a neuroplasticity 'signature' for each region. The dendritic spines of the motor cortex were found to be highly dynamic from P60 through to P90 in both male mice and females experiencing baseline levels of oestrogen, whilst during cycling peaks of oestrogen the dendritic spines of the motor cortex were observed to be stable. Conversely, the somatosensory cortex displayed early stability within males, prior to higher dynamics at adulthood as seen in females. These findings revealed that spine dynamics were specific to brain region, and that in the motor cortex dependent upon cycling oestrogen. Within the motor cortex of *TDP-43^{A315T}* mice at P60, males displayed significantly reduced dynamics, whilst high oestrogen females were associated with increased dendritic spine dynamics. Collectively, these findings indicate the sustained dynamics of the motor cortex in normal conditions may prime the region to fail in the presence of activity-dependent pathology at the dendritic spine. The potential for cycling oestrogen to be neuroprotective at the dendritic spine under these conditions is evident, and highlights the need for further studies to explore this as a potential therapeutic pathway.

The findings from this thesis provide strong evidence in support of the crucial role neuroplastic mechanisms at the dendritic spine have in response to pathology. Here, evidence is provided for the early dysfunction of the synapse in an ALS disease model being a key disease event within the presymptomatic motor cortex, a region that may be primed to fail fundamentally as a result of sustained activity demands at the dendritic spine. Further, the thesis supports the notion of oestrogen as being neuroprotective at the synapse, and indicates the transient peaks of the hormone maintain compensatory dynamics in the presence of misprocessed TDP-43. These results highlight the targeted nature of vulnerability to neurodegenerative diseases, and highlight the need to target early dysregulation of

neuroplasticity at the synapse to protect select cortical networks prior to neurodegeneration. Importantly, the identification of cycling oestrogen as being protective at the dendritic spine may be critical for future efforts aimed at developing new therapeutic targets in ALS and other neurodegenerative diseases; for this application to be viable, it is essential to investigate further the role for disease proteins at the dendritic spine, as well as the mechanisms through which oestrogen exerts neuroprotective effects.

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ABBREVIATIONS

2PLSM	2-photon laser scanning microscopy
ACSF	Artificial cerebrospinal fluid
ADAR2	Adenosine deaminases that act on RNA
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
AMPA	α-amino-3-hydroxyl-5-methylisoxazole-4-propionic acid
AMPAR	α-amino-3-hydroxyl-5-methylisoxazole-4-propionic acid receptor
BOLD	Blood-oxygen-level-dependent
C9orf72	Chromosome 9 Open Reading Frame 72
$CaCl_2$	Calcium chloride
Cacna1c	Voltage-dependent calcium channel
CaMKII	Calcium/calmodulin-dependent kinase
CLIP-seq	Crosslinking immunoprecipitation sequencing
CNS	Central nervous system
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
CTD	C-terminal domain
DAPI	4’6-diamidino-2-phenylindole
DPR	Dipeptide repeats
EAAT1	Excitatory amino acid transporter 1
EAAT2	Excitatory amino acid transporter 2
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
eIF4E	Eukaryotic translation initiation factor
ERβ	Oestrogen receptor beta
fALS	Familial amyotrophic lateral sclerosis
fMRI	Functional magnetic resonance imaging
FMRP	Fragile X mental retardation protein
FUS/TLS	Fused in sarcoma/translocated in liposarcoma
GABA	Gamma aminobutyric acid

GluA	Ionotropic glutamate receptor
GLT-1	Glutamate transporter 1
GTP	Guanosine 5′-triphosphate sodium salt hydrate
Hz	Hertz
IgG	Immunoglobulin G
IP	Intraperitoneal
iPSCs	Induced pluripotent stem cells
K^+	Potassium
KCl	Potassium chloride
KCNMA1	Calcium-activated potassium channel alpha
LCS	Low-complexity sequence
LMN	Lower motor neuron
LTP	Long-term potentiation
M	Molar
MAP1B	Futsch/microtubule associated protein b
MC	Motor cortex
mEPSC	Miniature excitatory postsynaptic current
mg	Milligram
MgATP	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
$MgCl_2$	Magnesium chloride
ml	Millilitre
mM	Millimolar
mm	Millimetre
mV	Millivolts
ms	Milliseconds
mOsm	Milliosmoles
nA	Nano-amps
Na^+	Sodium
NaCl	Potassium chloride
$NaHCO_3$	Sodium bicarbonate

NaH_2PO_4	Monosodium phosphate
NF	Neurofilament
nM	Nano molar
nm	Nano metre
NMDA	N-methyl-D-aspartate
NMJ	Neuromuscular junction
NPY	Neuropeptide Y
Nrxn	Neurexin
NTD	N-terminal domain
O^2	Oxygen
OH	High oestrogen
PBS	Phosphate buffered saline
pA	Pico amps
PCR	Polymerase chain reaction
pF	Pico farad
PFA	Paraformaldehyde
Prp	Prion-promoter
PSD	Post-synaptic density
PSD-95	Post-synaptic density 95
Rac1	Ras-related C3 botulinum toxin substrate 1
RAN	Non-AUG dependent
RBP	RNA-binding protein
ROI	Region of interest
RRM1	RNA-recognition motif 1
RRM2	RNA-recognition motif 2
sALS	Sporadic amyotrophic lateral sclerosis
SC	Subcutaneous
SD	Standard deviation
SEM	Standard error of the mean
Slap	SRC-like adaptor protein

SOD1	Superoxide dismutase 1
SSC	Somatosensory cortex
ssRNA	Single-stranded RNA
Stx1A	Syntaxin-1A
Syt17	Synaptotagmin
TEA	Triethylamine
TMS	Transcranial magnetic stimulation
TDP-43	Transactive response DNA binding protein 43
UMN	Upper motor neuron
VAMP1	Vesicle-associated membrane protein 1
WT	Wild-type
µL	Micro litre
µm	Micro metre
µM	Micro molar
^{o}C	Degrees Celsius
Ω	ohms

1 INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease that is presently incurable and manifests as the degeneration of the motor system (Cleveland & Rothstein 2001, Talbot 2014) (Figure 1.1.1). The disease is characterised by the loss of cortical upper motor neurons (UMNs) and spinal lower motor neurons (LMNs); the deterioration of this system then leads to muscle weakness, disability, paralysis and eventual death (Logroscino et al 2008) . The disease strikes 2/100, 000 people annually, with a male to female ratio of 1:5 for sporadic disease forms (Chio et al 2013). The mean age of onset is 55-60 years and with no treatments available to successfully halt disease progression, the majority of patients die within 2-5 years of diagnosis, primarily from respiratory failure (Al-Chalabi & Hardiman 2013, Chio et al 2013). Two drugs have been approved for the treatment of ALS; the first of these being Riluzole and the most recent being Edaravone (Abe et al 2017, Bensimon et al 1994, Cruz 2018, Fang et al 2018). Riluzole is a glutamatergic antagonist, associated with a 35% reduction in mortality and being particularly efficacious at the later stages of disease (Fang et al 2018). The exact mechanism of action of edaravone treatment in ALS is still unknown, though therapeutic benefits may be a result of antioxidant properties (Cruz et al 2018).

ALS is a multifactorial disorder and this translates into vast clinical heterogeneity. Disease onset is focal, and patients experience fatigue in conjunction with limb onset (weakness and wasting of the limbs) or bulbar onset (tongue fasciculation) (Hardiman et al 2011, Turner & Swash 2015). The focal nature of onset then becomes something else entirely, following the systematic spread of pathology throughout the motor system- culminating in respiratory failure and death (de Carvalho et al 1996). Pathological spread from a focal point to canonical regions is indicative of common mechanisms in select regional vulnerability; thus to halt or prevent the spread of pathology throughout cortical regions, the earliest disease events need to be identified to produce therapeutic targets (Figure 1.1.2). While the majority of ALS cases are sporadic (sALS), with ten percent being linked to familial mutations (fALS), the overlap in disease presentation in both sporadic and heritable disease further indicates shared neurodegenerative pathways (Pochet 2017).

In 2006 the transactive response DNA-binding protein 43 (TDP-43) was identified as the primary component of intracellular ubiquitinated inclusions that characterise 97% of sporadic ALS cases, and familial disease forms have been linked to mutations in the TARDBP gene that encodes for TDP-43 (Geser et al 2009, Ling et al 2013, Neumann et al 2006, Umoh et al 2018). Furthermore, specific neuronal networks are spared in sporadic disease and even in the presence of ubiquitously expressed heritable mutations; cognitively, higher order executive functions, problem solving and memory are

Figure 1.1.1

Schematic of the motor system, selectively vulnerable in ALS. The system is composed of upper motor neurons in the motor cortex, and bulbar and spinal (lower) motor neurons within the spinal cord which innervate skeletal muscles. Reproduced from Brown, 2017.

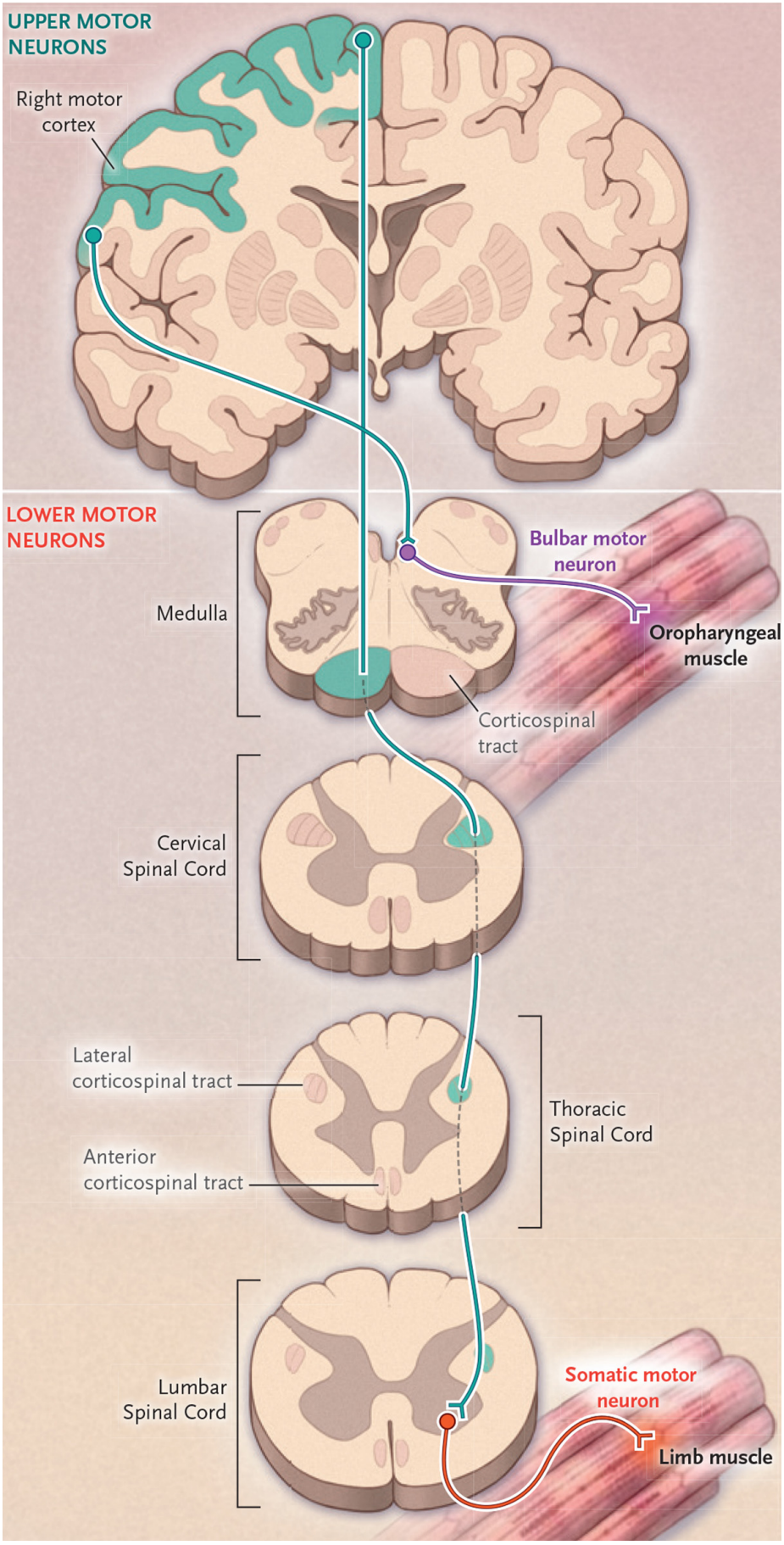
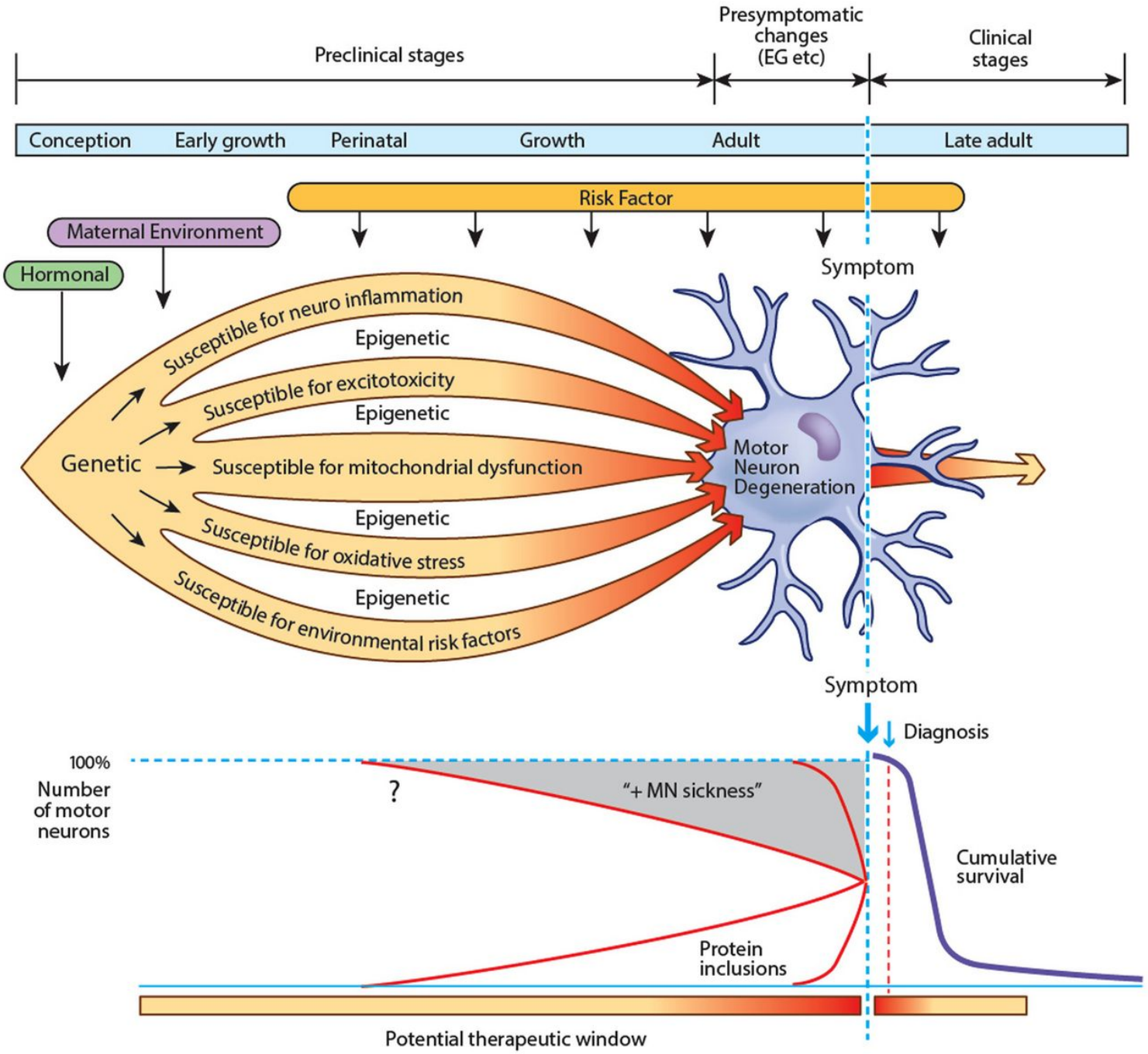

UPPER MOTOR NEURONS
Right motor cortex
LOWER MOTOR NEURONS
Medulla
Bulbar motor neuron
Oropharyngeal muscle
Corticospinal tract
Cervical Spinal Cord
Lateral corticospinal tract
Anterior corticospinal tract
Thoracic Spinal Cord
Lumbar Spinal Cord
Somatic motor neuron
Limb muscle

Figure 1.1.2

The development of ALS may begin during a prolonged pre-clinical time period. Motor neurons are inherently susceptible to inflammation, excitotoxicity, mitochondrial dysfunction and environmental risk factors. Epigenetics may also further influence sensitivity and susceptibility to these insults. The high activity-load of motor neurons leads to metabolic stress and progressive loss of motor neuron function; after a prolonged period of compensation, these mechanisms may then begin to fail and early presymptomatic features such as electrophysiological abnormalities may be detectable. By clinical manifestation of disease, the motor system fails and ALS becomes progressively symptomatic. Reproduced from Eisen et al 2014.


Presymptomatic changes (EG etc)
Preclinical stages
Clinical stages
Conception
Early growth
Perinatal
Growth
Adult
Late adult
Risk Factor
Maternal Environment
Hormonal
Symptom
Susceptible for neuro inflammation
Epigenetic
Susceptible for excitotoxicity
Epigenetic
Genetic
Susceptible for mitochondrial dysfunction
Epigenetic
Susceptible for oxidative stress
Epigenetic
Susceptible for environmental risk factors
Motor Neuron Degeneration
Symptom
Diagnosis
100%
Number of motor neurons
?
"+ MN sickness"
Cumulative survival
Protein inclusions
Potential therapeutic window

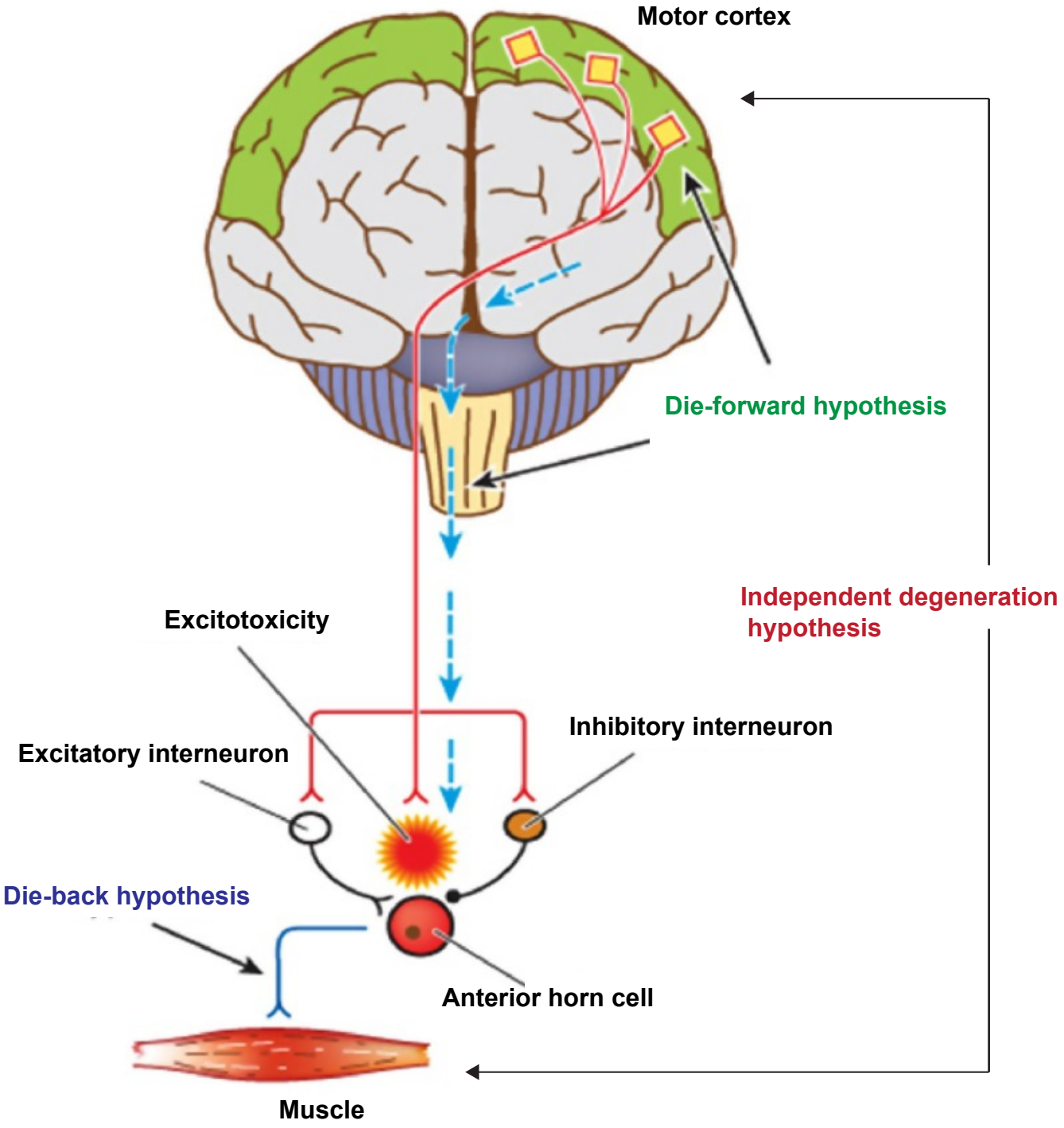
preserved, whilst bladder control and eye movement are unaffected by the pathological spread of motor neuron neurodegeneration (Bruijn & Cudkowicz 2014, Proudfoot et al 2016). Together, this suggests that not only is TDP-43 dysfunction intimately associated with ALS pathogenesis, it is also implicated in the differential vulnerability of cortical regions.

1.1 EPIDEMIOLOGY AND CLINICAL MANIFESTATION

The prevalence of ALS in Australia is thought to be 8 sufferers per 100 000, with approximately 1500 patients living with the disease at a given time (Kiernan et al 2011). With no biomarkers or tests currently widely available, diagnosis of ALS is based on clinical scoring using neuropsychological tests and structural brain imaging (Hardiman et al 2011). Both fALS and sALS share the focal degeneration of motor neurons, grouped into upper populations of the motor cortex and the lower populations of the spinal cord and brain stem (Fogarty 2018). As these populations degenerate, the synaptic connectivity with target muscles is lost, leading to atrophy of the muscle and characteristic manifestation of disease (Brown & Al-Chalabi 2017). The loss of UMN populations induces muscle stiffness and spasticity, whilst LMN loss is observed through excessive excitation that induces fasciculations, observed as muscle spasms (Al Chalabi et al 2011). Approximately 70% of patients will present with limb onset, 25% with bulbar onset (speech and swallowing difficulties), and 5% with initial respiratory involvement that ultimately spreads to other regions (Kiernan et al 2011). Though it is apparent that the motor system as a whole is affected in ALS, it is still unclear as to where the initial pathological insult begins; multiple hypotheses attempt to cohesively understand the timing and extent of UMN and LMN degeneration. The ‘dying-back’ hypothesis has historically been the most accepted, and encompasses initial periphery neuronal loss prior to retrograde degeneration toward the cortex (Dadon-Nachum et al 2011, Fischer et al 2004, Williamson & Cleveland 1999). Conversely, the ‘dying-forward’ hypothesis concerns disease initiation in the motor cortex, followed by anterograde neurodegeneration and loss of motor network connectivity (Eisen et al 1992). An alternative, third hypotheses has also gained traction in recent literature- that of independent degeneration. This theory suggests neurodegeneration occurs at both ends of the motor system near-simultaneously, resulting in bi-directional neuronal loss, and highlights the importance of treating the circuitry of the motor system as a whole (Dervishi & Ozdinler 2018, Grad et al 2017) (Figure 1.1.3). Contentious as these concepts may be, increasing evidence indicates the early dysfunction of upper motor neurons in the motor cortex precedes the clinical manifestation of disease prior to onset, indicating the cortex is an early site of pathology (Geevasinga et al 2016a, Menon et al 2015, Ozdinler et al 2011, Vucic et al 2011). Identifying the earliest disease mechanisms is essential for finding an efficacious cure; however, due to the diversity in disease manifestation it has proven incredibly difficult to identify therapeutic and

Figure 1.1.3

Schematic of the die-forward (green text), die-back (blue text) and independent degeneration (red text) hypotheses of ALS. The die-forward hypothesis posits that ALS onset begins within the corticomotor system, with lower motor neuron degeneration resulting later in the cascade due to glutamate-mediated excitotoxicity. The die-back hypothesis posits that ALS begins within muscles, at the neuromuscular junction. The disease cascade then travels retrogradely to the cell body and towards the corticomotor system. In contrast, the independent-degeneration hypothesis suggests both upper and lower motor neurons degenerate independently and concurrently within their distinct compartments. Reproduced from Vucic et al 2013.


Motor cortex
Die-forward hypothesis
Independent degeneration hypothesis
Excitotoxicity
Inhibitory interneuron
Excitatory interneuron
Die-back hypothesis
Anterior horn cell
Muscle

neuroprotective targets, and diagnosis is often complex and delayed (Geevasinga et al 2016b) (Figure 1.1.2). A major barrier to understanding ALS has been the inability to create viable disease models to understand the complex combination of pathological pathways and rare occurring de novo mutations.

1.1.1 Familial ALS and Protein Dysfunction

A theme common in ALS is the dysfunction and aggregation of several protein species, both mutant and wild-type, that undergo distinct posttranslational modifications (see Table 1.1.1). The first gene and protein whose mutations were identified as ALS-causing was cytosolic superoxide dismutase [Cu/Zn] (SOD1) (Rosen et al 1993). The majority of missense and truncation protein mutations induce SOD1 inclusion bodies within spinal motor neurons, with a correlation between the degree of conformational instability and rate of disease progression (Borchelt et al 1994, Prudencio et al 2009). The molecular underpinnings of SOD1 cytotoxicity disrupt an array of normal functions, partly associated with mechanisms of oxidative stress and the aberrant trafficking of copper and zinc; this is in addition to disrupted mitochondrial trafficking, impaired molecular motors and axonal transport, and interference with neuromuscular synapses (Beckman et al 1993, Estevez et al 1999, Morfini et al 2013, van Zundert et al 2012, Vande Velde et al 2011). The creation of the $SOD1^{G93A}$ mouse model- expressing high levels of mutant protein- has been crucial for investigations into ALS through the recapitulation of pathological and clinical phenotypes observed in human cases (Gurney et al 1994).

A major breakthrough in investigating the aggregation of ubiquitinated proteins as an early and canonical feature of ALS pathology came with the identification of pathological TDP-43 inclusions, along with mutations in the gene encoding for the protein in sALS and fALS (Neumann et al 2006). The protein TDP-43 has been identified as a pathological hallmark in a range of disparate neurodegenerative diseases, with the depletion and increase of protein levels implicated in the progression of both sporadic and familial ALS (D'Alton et al 2014, Gendron & Petrucelli 2011). The fused in sarcoma/translocated in liposarcoma protein (FUS/TLS) is an RNA-binding protein that has also been implicated in ALS through heritable gene mutations (Kwiatkowski et al 2009, Vance et al 2009) (Dormann et al 2010). FUS/TLS is structurally and functionally similar to TDP-43, with mutations identified in a range of intracellular inclusions (Dobra et al 2018).

An advance in identifying the genes that underlie fALS came in 2011, with the discovery of 40-50% of cases being attributed to mutations in the *C9ORF72* gene (DeJesus-Hernandez et al 2011, Renton et al 2011). Normally, the C9ORF72 (Chromosome 9 Open Reading Frame 72) protein has roles in trafficking nuclear/endosomal membranes and autophagy, featuring a noncoding stretch of six nucleotides repeats up to 30 times; however, in ALS this segment is repeated hundreds to thousands

Table 1.1.1 Adapted from Taylor et al 2016.

Locus	Gene	Protein	Function	Mutations	Proportion of ALS		Discovery
					Familial	Sporadic	
21q22.1	SOD1	Cu/Zn superoxide dismutase	Superoxide dismutase	>150	20%	2%	Rosen et al 199
2p13	DCTN1	Dynactin	Component of dynein motor complex	10	1%	<1%	Puls et al 2003
14q11	ANG	Angiogenin	Ribonuclease	>10	<1%	<1%	Greenway et a 2006
q36	TARDBP	TDP-43	RNA-binding protein	>40	5%	<1%	Kabashi et a 2008; Sreedharan et a 2008
16p11.2	FUS	FUS/TLS	RNA-binding protein	>40	5%	<1%	Kwiatkawski e al 2009; Vanc et al 2009
9p13.3	VCP	Valosin-containing protein	Ubiquitin segregase	5	1-2%	<1%	Johnson et a 2010
10p15-p14	OPTN	Optineurin	Autophagy adaptor	1	4%	<1%	Maruyama et a 2010
9p21-22	C9ORF72	C9ORF72	Possible guanine nucleotide exchange factor	Intronic GGGGCC repeat	25%	10%	De-Jesus Hernandez et a 2011; Renton e al 2011
Xp11.23-Xp13.1	UBQLN2	Ubiquilin2	Autophagy adaptor	5	<1%	<1%	Deng et al 201
5q35	SQSTM1	p62	Autophagy adaptor	10	<1%		Fecto et al 201 Teyssou et a 2011
17p13.2	PFN1	Profilin-1	Actin-binding protein	5	<1%	<1%	Wu et al 2012
12q13.1	HNRNPA1	hnRNPA1	RNA-binding protein	3	<1%	<1%	Kim et al 201 Liu et al 2013
5q31.2	MATR3	Matrin3	RNA-binding protein	4	<1%	<1%	Johnson et a 2014
2q36.1	TUBA4A	α-tubulin 4 a	Microtubule subunit	7	<1%	<1%	Smith et al 201
22q11.23	CHCHD10	Coiled-coil-helix-coiled-coil-helix domain containing 10	Mitochrondrial protein of unknown function	2	<1%	<1%	Bannwarth et a 2014
12q14.1	TBK1	TANK-binding kinase 1	Regulates autophagy and inflammation	10	?	?	Freischmidt e al 2015
16p13.3	CCNF	Cyclin F	Ubiquitin proteolysis	10	?	?	Williams et a 2016
4q33	NEK1	Never in mitosis gene A-related kinase1	Cell cycle control and cilia regulation	4	?	?	Brenner et a 2016; Kenna e al 2016
21q22.3	C21ORF2	Cilia and flagella-associated protein 410	Ciliogenesis and DNA damage repair	21	?	?	van Rheenan e al 2016; Iye etal 2018
2p13.3	TIA1	Cytotoxic granule-associated RNA-binding protein	RNA-binding protein, component of stress granules	1	2%	0.4%	Mackenzie et a 2017
10q22.3	ANXA11	Annexin A11	Calcium-dependent phospho-lipid binding protein	3	?	?	Smith et al 201

of times, with three mechanisms thought to induce disease state. A loss of function mechanism has been theorised to be caused by down-regulation of *C9ORF72* gene expression (Sakkas et al 2017). Secondly, a gain-of-function mechanism has been associated with the sequestration of RBPs into intranuclear foci (Kumar et al 2017). The third proposed mechanism is that of dipeptide-repeat protein (DPR) production through abnormal non-AUG-dependent (RAN) translation of RNAs containing repeats (Freibaum et al 2015, Zhang et al 2015). While mouse models of *C9ORF72* mutations recapitulate the molecular signature of associated ALS, most of these mice do not show a strong motor phenotype, with the exception of the BAC mouse model (Liu et al 2016, O'Rourke et al 2016, Peters et al 2015).

Models of fALS mutations have been used to uncover a swathe of shared pathological pathways, as well as mutation-specific phenotypes that have broadened our knowledge of shared mechanisms that may be relevant across all forms of ALS. To better understand incidences of ALS outside of heritable mutations, research models also need to encompass sporadic pathological pathways.

1.1.2 Sporadic insights

Though family history in sporadic disease is absent, twin studies demonstrate heritability is still ~60% and mutations identified in fALS can also be found in sALS (Al-Chalabi et al 2010). This is confounded by fALS mutations manifesting as other neurodegenerative disorders; the intermediate penetrance of several fALS gene variants; and the combination of variants that may increase the risk of ALS through apparently sporadic mechanisms (Andersen & Al-Chalabi 2011, Cirulli et al 2015, Freischmidt et al 2015, Pottier et al 2015, van Blitterswijk et al 2012). Still, studies of heritability cannot account for all sALS cases and these are thought to be a result of undefined factors or environmental exposures. Studies of environmental factors have been inconclusive and are notoriously fraught due to the large number of possible exposures, and the temporal difficulty of identifying when exposures occurred. Additionally, poor prognosis is linked to reporting bias, wherein the patients who survive long enough to contact specialist research centres differ from those included in population studies (Lacorte et al 2016). Despite these issues, factors such as military service, smoking, and chronic exposure to heavy metals and electromagnetic fields have been associated with ALS in multiple studies (Beard et al 2016, Ingre et al 2015, Wang et al 2011).

In both sALS and fALS, it is apparent that the motor system is uniquely susceptible to the disease mechanisms that characterise ALS. It is critical to identify the pathological pathways shared by fALS and sALS to understand the select vulnerability of the motor cortex to this disease.

1.2 THE DIFFERENTIAL VULNERABILITY OF CORTICAL REGIONS IN DISEASE

Early synaptic dysfunction within vulnerable cortical regions has been identified across a range of neurodegenerative diseases (Henstridge et al 2016). The human brain constructs and functions via intricate large-scale neural connectomes that are critically dependent on functional feedback at the synapse. These discrete regions are defined by synchronous baseline activity; a unified and stepwise reaction to stimulation; and selective vulnerability to neurodegenerative disorders (Seeley et al 2007, Henstridge et al 2016). Accordingly, correlated spontaneous activity occurs within functionally related and spatially distinct groups of cortical and subcortical regions (Vincent et al 2007). These direct or indirect anatomical connections exist within intrinsic connectivity networks, as evidenced by functional magnetic resonance imaging (fMRI) and blood-oxygen-level-dependent (BOLD) signal fluctuations during sleep, and during mental effort (Fransson et al 2007, Greicius et al 2009, Hesselmann et al 2008, Raichle 2009). Neurodegenerative diseases induce progressive and debilitating dysfunction as a result of localised regional pathology, and a canonical pattern in disease is the misfolding of key proteins within targeted neuronal populations residing in distinct brain regions (Seeley et al 2009, Takalo et al 2013, Walker et al 2013). As pathology spreads throughout interconnected anatomical areas, synaptic connections within intrinsic networks falter and are accompanied by a worsening in disease manifestation (Buckner et al 2009, Greicius et al 2004, Palop et al 2006, Selkoe 2002).

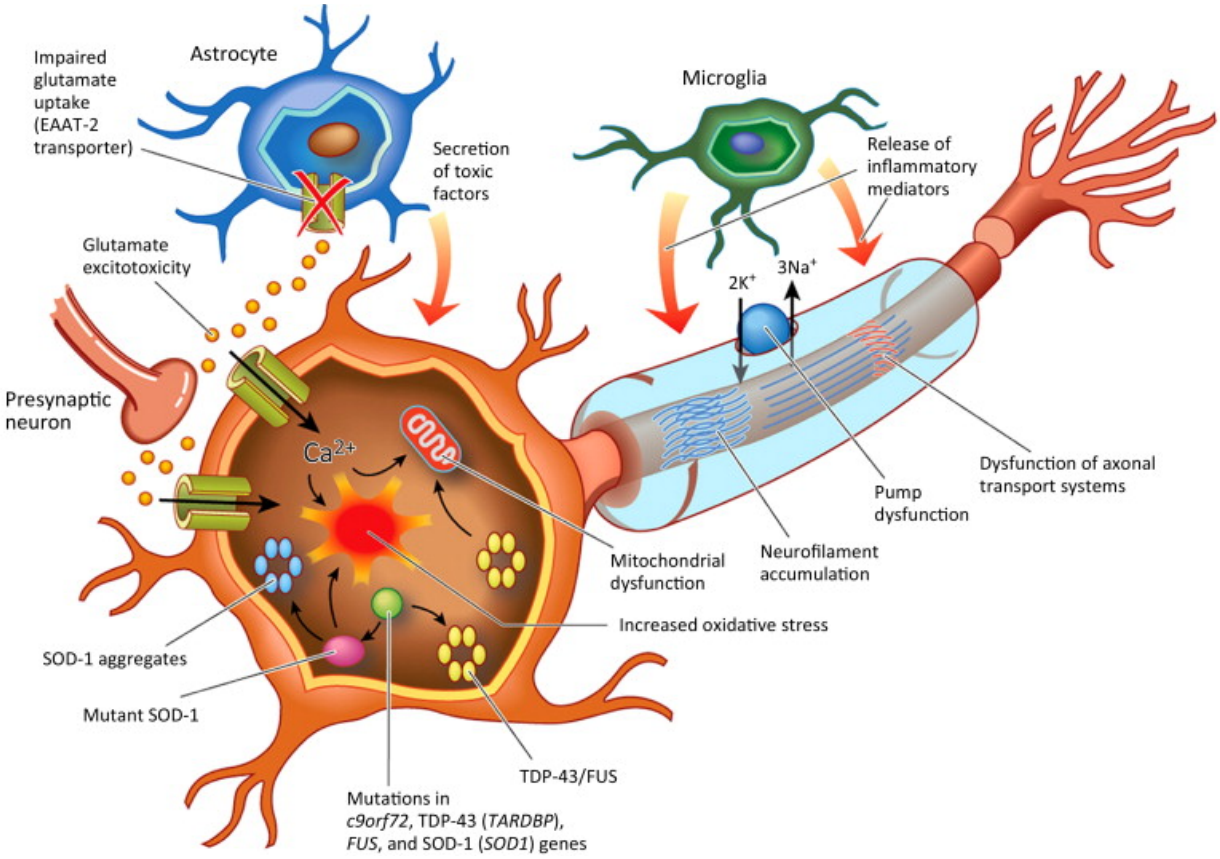
ALS features the exclusive dysfunction of the motor system, with pathology spreading to the frontal and temporal lobes in later disease stages (Brettschneider et al 2013). Within the cortex, UMNs are selectively lost in a unique pattern of degeneration (Nihei et al 1993); though there is increasing recognition of other affected neuronal subgroups in the disease, particularly after a prolonged disease course. While synaptic dysfunction has been increasingly implicated in the disease, a myriad of pathological intrinsic and extrinsic mechanisms have been identified as specifically impacting motor neurons during ALS progression.

1.2.1 Motor neuron pathology

The clinical manifestation of ALS is distinct, yet the complexity of molecular mechanisms implicated in this multifactorial disease underlie vast heterogeneity. Various neurochemical and molecular pathways of motor neurons render cells vulnerable to insults, and in ALS genetic factors, glutamatergic toxicity, oxidative stress and damage to specific proteins and organelles may all be important contributory factors (Shaw & Eggett 2000) (Figure 1.1.2; Figure 1.1.4). Motor neurons differ vastly

Figure 1.1.4

The pathophysiological mechanisms underlying neurodegeneration in ALS. The disease is multifactorial, and involves molecular and genetic pathways. Astrocytic dysfunction is associated with reduced glutamate uptake by excitatory amino acid transporter 2 (EAAT2) from the synaptic cleft, and is linked to excitotoxicity. Glutamate-induced excitotoxicity results in the influx of Na^+ and Ca^{2+} ions, with neuronal death due to activation of Ca^{2+} enzymatic pathways and the generation of free radicals. Mutations in key genes such as C9ORF72, TARDBP and FUS induce dysregulation of RNA processing and metabolism, linked to protein mislocalisation and toxic cytoplasmic inclusions. SOD1 mutations increase oxidative stress and mitochondrial dysfunction, as well as affecting neurofilamental and axonal transport processes. Secretion of proinflammatory cytokines follows microglial activation. Adapted from Vucic et al 2014.


Impaired glutamate uptake (EAAT-2 transporter)
Astrocyte
Secretion of toxic factors
Microglia
Release of inflammatory mediators
Glutamate excitotoxicity
2K+
3Na+
Presynaptic neuron
Ca2+
Dysfunction of axonal transport systems
Pump dysfunction
Mitochondrial dysfunction
Neurofilament accumulation
Increased oxidative stress
SOD-1 aggregates
Mutant SOD-1
TDP-43/FUS
Mutations in c9orf72, TDP-43 (TARDBP), FUS, and SOD-1 (SOD1) genes

from other cells in somatic diameter and the length of axonal processes; consequently, this comes with increased energy, metabolic and mitochondrial demands, along with a high content of neurofilament proteins (Gao et al 2009).

Mitochondrial alterations have been observed in patient tissue and in animal models, with impairments in organelle clearance and calcium buffering, apoptosis and defective mitochondrial transport (Cozzolino & Carrì 2012, Cozzolino et al 2013, Palomo & Manfredi 2015). The majority of studies implicating mitochondrial involvement in ALS come from those on SOD1-associated disease forms with SOD1 mutations increasing protein accumulation in mitochondria, inducing defective function, mitochondrial damage and ALS-like phenotypes (Cozzolino et al 2009, Magrané et al 2009, Pickles et al 2016, Tafuri et al 2015). The translocation of mutated FUS to mitochondria also seems to play a role in this pathway, interacting with the mitochondrial chaperone HSP60 and inducing an ALS phenotype in *Drosophila* expressing disease-associated mutations (Deng et al 2015). The increased demands required to sustain motor neurons are clearly factors in the underlying susceptibility to disease, and this extends to the cytoskeletal components of the cell.

The axonal length of motor neurons necessitates relatively high neurofilament content, with neurofilament (NF) proteins being the major component of the cytoskeleton (Hirokawa et al 1984, Oberstadt et al 2018). A feature of ALS is the abnormal assembly and accumulation of neurofilaments within the cell body and axons of motor neurons; though it is not known whether these events are a consequence of blocked axonal transport, or whether protein accumulation mediates secondary impairment (Hirano 1991). In human embryonic stem cells expressing a SOD1 mutation, NF misregulation has been found to mediate aggregation and axonal degeneration in ALS-patient derived motor neurons (Chen et al 2014). Aberrant neurofilaments are also found localised with ubiquitinated TDP-43 inclusions within the cytoplasm, with variations in the subunits of NFs (Menke et al 2015, Turner & Gray 2016).

Gene mutations associated with ALS impact multiple cell types, and it is now apparent the disease arises partly from non-cell-autonomous mechanisms (Taylor et al 2016). Microglia are the immune cells of the nervous system, and are activated in ALS; indeed, microglial synthesis of mutant SOD1 is a determinant of rapid disease progression (Boillee et al 2006). In C9ORF72 variants of ALS, the inactivation of the protein encoded by *C9ORF72* results in abnormal microglia and progressive inflammation, providing further evidence towards microglia-mediated inflammation in ALS (Burberry et al 2016, O'Rourke et al 2016). Astrocytes are another glial cell-type implicated in non-cell autonomous pathology, whereby astrocytes affected by ALS mutants are implicated in diffusible motor

neuron toxicity (Di Giorgio et al 2007, Re et al 2014). These cells play a critical role in governing neuronal transmission- responsible for neurotransmitter clearance at the synapse and critical for the protection of neurons from overstimulation (Malarkey & Parpura 2008, Schousboe et al 2014). The deregulation of glutamate metabolism and clearance has been identified through increased glutamate in the cerebrospinal fluid (CSF) of ALS patients, whilst studies in ALS post-mortem tissue and SOD1 mice have identified reductions in excitatory amino acid transporters (EAAT1 and EAAT2), and in glutamate transporters (GLT-1) (Lin et al 1998, Pardo et al 2006, Rothstein et al 1990, Rothstein et al 1995). Though a range of pathways are implicated here in motor neuron susceptibility in disease, accumulating research now suggests the dysregulation of glutamate and excitation may be critical to the onset and progression of neurodegenerative mechanisms.

1.2.2 Cortical Excitability

A well-documented aspect of ALS pathophysiology is cortical hyperexcitability- an increased neuronal or network response to a stimulus (Bae et al 2013, Do-Ha et al 2018). Glutamate is the principle excitatory neurotransmitter of the brain, with three main ionotropic receptors; N-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methylisoxazole-4-propionic acid (AMPA) and Kainite (Meldrum 2000). Glutamatergic transmission is essential for cortical plasticity and long-term potentiation, yet can also promote excitotoxic environments that can lead to neuronal loss within cortical networks (Kalia et al 2008). Investigating neurophysiological network features in fALS and sALS cohorts has shown hyperexcitability is a common pathology, and accordingly patients often display early increases in neural activity indicative through muscle cramping and fasciculations, months before the clinical diagnosis of the disease (Bae et al 2013, de Carvalho et al 2014, Mills & Nithi 1997, Vucic et al 2008, Wainger et al 2014). Several clinical methods can be employed to investigate changes to excitation within cohorts *in vivo*, including transcranial magnetic stimulation (TMS) (de Carvalho et al 2008). TMS stimulates the primary motor cortex, producing electrical currents and measurable muscle responses (Di Lazzaro et al 2006, Nurmikko et al 2016, Treister et al 2013). Using this technology, studies have shown patients in the early stages of ALS require a smaller stimulus to generate an evoked response, indicating a hyperexcitable motor cortex (Menon et al 2015, Vucic & Kiernan 2006). The drug Riluzole is known to slow ALS progression- though only by a few months- and a proposed pathway for efficacy is the inhibition of persistent sodium (Na^+) channels, though any effects on hyperexcitability are transient (Geevasinga et al 2016a, Kuo et al 2005, Pieri et al 2009, Vucic et al 2013a). Additionally, the reduction of potassium (K^+) channels has been confirmed in patient cohorts, potentially contributing to hyperexcitability and fasciculations (Jiang et al 2005, Kanai et al 2006, Shibuya et al 2011).

The inhibitory networks of the cortex can provide further insights into cortical hyperexcitability. To balance excitation within neural circuitry, inhibitory interneurons utilise gamma-aminobutyric $acid_A$ (GABA)- the predominant inhibitory neurotransmitter in the brain (Buzsáki & Wang 2012, Owens & Kriegstein 2002). Applying TMS to ALS patients has shown the threshold to produce motor-evoked potentials is reduced following an inhibitory stimulus, particularly so in those with less severe symptomology. This suggests that not only is cortical excitability present early in disease, it may be reduced at later progressive stages (Floyd et al 2009, Vucic et al 2018, Vucic et al 2013b). Furthermore, specific interneuronal populations are perturbed, whereby the loss of calretinin interneurons in the $SOD1^{G93A}$ mouse and fluctuating levels of neuropeptide-Y (NPY) interneurons may reflect compensatory mechanisms of the motor circuitry (Clark et al 2017). In layer V of the motor cortex, a decrease in interneuron populations occurs concurrently with a reduction in the frequency of GABA receptor-mediated postsynaptic currents, leading to cortical hyperexcitability (Nieto-Gonzalez et al 2011).

Hyperexcitability is associated with the exaggerated influx of calcium through AMPA receptors (AMPARs), and alterations to key synaptic receptors are linked to toxic alterations in calcium permeability (Hardingham & Bading 2010, Kawahara et al 2004, Kwak & Kawahara 2005, Lau & Tymianski 2010). Motor neurons express abundant glutamate receptors and as such, the cell type is especially vulnerable to AMPAR-mediated excitotoxicity (Carriedo et al 1996, Krieger et al 1994, Van Den Bosch et al 2000). The calcium permeability of AMPARs is largely mediated by the ionotropic glutamate receptor 2 (GluA2) subunit, and the process of post-transcriptional editing to induce calcium impermeability at the Gln/Arg site (Grosskreutz et al 2010, Kawahara et al 2004, Sasaki et al 2015, Yamashita & Kwak 2014b). The low expression of GluA2 and calcium-buffering proteins render motor neurons uniquely vulnerable to excitability of the cortex in ALS, though clear evidence indicating that it is the primary disease mechanism is lacking.

Intriguingly, recent studies have now shown that the most vulnerable motoneuron populations lose the ability to fire repetitively- a signature of hypoexcitability (Devlin et al 2015, Martinez-Silva et al 2018). These insights have challenged the notion of hyperexcitability being responsible for cell death, evidenced by the resilience of hyperexcitable subsets of motor neurons, and the protection of motor neurons from ALS-linked pathology following induction of excitability (Leroy & Zytnicki 2015, Saxena et al 2013). Studies utilising ALS human induced pluripotent stem cells (iPSCs) have indicated evidence for both hypo- and hyperexcitability; however, some light has been shed on these contradictory findings from a 2015 study, where a switch from initial hyperexcitability to later hypoexcitability was reported in motor neurons differentiated from patient-derived iPSCs harbouring

TARDBP and C9ORF72 gene mutations (Naujock et al 2016). The susceptibility of motor neurons to hypoexcitability has been further elucidated following the identification of the most vulnerable populations being larger motor units containing fast-contracting muscle fibers, breaking down at a faster rate than slower motor units. Furthermore, these large motor units display an inability to fire repetitively even though their neuromuscular junctions are still functional (Martinez-Silva et al 2018). Together, these studies suggest intrinsic hyperexcitability is unlikely to be the major cause of motor neuron degeneration, and that hypoexcitability may be a key player in the progression of ALS. Though we have evidence of changes in the excitability of neurons within the motor system, we still do not understand the mechanisms driving these changes; nor do we know when these insults are occurring over a timeline of disease. The misprocessing of the protein TDP-43 is another hallmark feature of ALS that is shared across the vast majority of cases, and the potential for an association between this and excitability may be key to identifying early disease mechanisms.

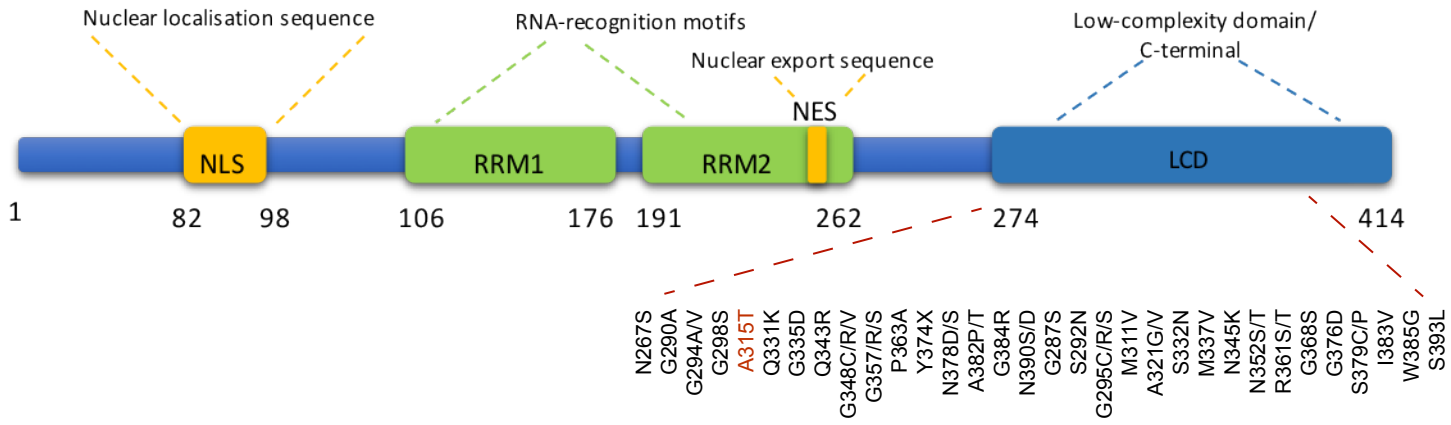
1.3 THE FUNCTIONS OF TDP-43

The hallmark characteristic of ALS is the aggregation and mislocalisation of TDP-43. The protein is an auto-regulating and widely expressed RNA-binding protein (RBP) localised to the neuronal nucleus, able to shuttle into the cytoplasm to perform functions in both compartments (Ayala et al 2008, Neumann et al 2006). The protein features two RNA-recognition motifs (RRM1 and RRM2), an N-terminal domain (NTD) and a glycine-rich C-terminal domain (CTD) (Neumann et al 2006). RRM1 has a crucial role in the binding affinity between TDP-43 and single stranded RNA (ssRNA), and both RRMs are essential for the regulation of transcription and RNA-splicing (Maurel et al 2018). The NTD encompasses the nuclear localisation signal and RRM1, and this domain is highly conserved between species (Kuo et al 2014, Mompean et al 2017, Yang et al 2010a)(Figure 1.1.5). Physiologically, the NTD is able to reversibly assemble full-length TDP-43, enhancing the functions of the CTD by raising the local concentration of these disordered domains (Mompeán et al 2017). The CTD is essential for protein-protein interactions, enabling liquid-liquid phase separation and aggregation (Buratti et al 2005, Conicella et al 2016, Kato et al 2012, Kuo et al 2009). The domain also facilitates reversible RNA granule assembly and soluble protein aggregation (Han et al 2012).

The structure of TDP-43 is essential for other roles in the regulation of mRNA splicing, translation and transport (Alami et al 2014, Bose et al 2011, Liu-Yesucevitz et al 2014, Polymenidou et al 2011, Tollervey et al 2011); the processing of micro RNAs via Drosha and Dicer complexes (Kawahara & Mieda-Sato 2012, Ling et al 2010); the assembly of stress granules to protect mRNA (Aulas & Vande Velde 2015, Liu-Yesucevitz et al 2010); and the trafficking of RNA granules to distal processes (Alami

Figure 1.1.5

The structure of TDP-43 and ALS-linked mutations. TDP-43 consists of a nuclear localisation sequence (NLS) and a nuclear export sequence (NES), two RNA recognition motifs (RMM1, RMM2) and a glycine-rich, low complexity sequence C-terminal domain (LCD). The mutations identified in TDP-43 in sporadic and familial ALS patients are primarily localised within the LCD. The mutation studied in the current thesis is highlighted in red (A315T).


Nuclear localisation sequence
RNA-recognition motifs
Nuclear export sequence
NES
Low-complexity domain/
C-terminal
NLS
RRM1
RRM2
LCD
1
82
98
106
176
191
262
274
414
N267S
G290A
G294A/V
G298S
A315T
Q331K
G335D
Q343R
G348C/R/V
G357/R/S
P363A
Y374X
N378D/S
A382P/T
G384R
N390S/D
G287S
S292N
G295C/R/S
M311V
A321G/V
S332N
M337V
N345K
N352S/T
R361S/T
G368S
G376D
S379C/P
I383V
W385G
S393L

et al 2014, Coyne et al 2015, Gopal et al 2017, Liu-Yesucevitz et al 2014). Upon stimulation, TDP-43 colocalises and co-purifies with other RBPs such as Staufen, and localised to dendrites within RNA-granules containing target mRNAs (Freibaum et al 2010, Yu et al 2012). The number of TDP-43-containing granules increases following neuronal activation and the protein is translocated to dendritic spines, establishing TDP-43 as a neuronal activity-response factor, particularly as a component of these granules (Alami et al 2014, Wang et al 2008a). Distal TDP-43 localisation is further supported by identification of the protein at the presynaptic membrane of neuromuscular junction (NMJ); a synaptic connection formed between motor neurons and muscle fibers (Narayanan et al 2013). Knowledge of these roles is critical for understanding how either the loss of or the gain of protein function underpins disease pathogenesis, and is achievable only through our ability to model TDP-43-mediated toxicity.

1.3.1 Investigating TDP-43 Toxicity

The pathological accumulation of TDP-43 was first identified in post-mortem patient tissue in 2006, yet the role of this pathology is still poorly understood in the context of human clinical features, outcomes and underlying molecular mechanisms. Autopsy cohorts have provided an unmatched opportunity to investigate clinical associations with aggregate pathology, and give further insights into the toxicity of TDP-43 misprocessing. The burden of insoluble TDP-43 has been associated with a greater severity of cognitive changes and a reduction in cortical thickness, affecting regions outside of the motor system by end stage (Brettschneider et al 2014, Ferraro et al 2018, Geser et al 2009, McCluskey et al 2009). The distinct aggregate patterning of TDP-43 has also been associated with disease phenotypes, whereby cortical dystrophic neurites indicate a poorer prognosis - despite the less severe loss of lower motor neurons (Takeuchi et al 2016). Interestingly, the burden of TDP-43 in the hypoglossal nucleus has been shown to discriminate ALS patients from other disorders with 98% accuracy, whilst TDP-43 burden in layer V pyramidal neurons in the anterior cingulate cortex is specifically linked with sporadic ALS, connecting regional protein pathology with distinct clinical phenotypes (Braak & Del Tredici 2018, Braak et al 2017, Tan et al 2015).

Though post-mortem tissue has proven an invaluable resource, the caveat remains that these cases are analysed at the end of the ALS disease course, and cannot shed light on the early or initiating mechanisms if the disease. The ability to generate human iPSCs from somatic cells allows the analysis of individual human phenotypes within a physiological context (Dimos et al 2008, Takahashi et al 2007). Derived motor neurons from both fALS and sALS patients demonstrate these cells are able to functionally mature, and recapitulate key disease aspects such as neuronal degeneration and insoluble protein accumulation (Burkhardt et al 2013, Egawa et al 2012, Guo et al 2017). Nucleocytoplasmic

deficits have been identified under conditions of misprocessed TDP-43 in the presence of C9ORF72 mutations, and sporadic lines have been used to identify mitochondrial involvement in disease; thus demonstrating the use for iPSCs to delineate pathogenic events in ALS (Alves et al 2015, Burkhardt et al 2013, Zhang et al 2016). Interestingly, regardless of genotype TARDBP and C9ORF72 mutations display initial hyperexcitability followed by progressive reductions in action potential outputs and synaptic activity, further supporting the early dysfunction of excitability as a common mechanism underlying motor neuron death in ALS (Devlin et al 2015).

While human tissue and iPSCs provide new pathways through which to investigate ALS and TDP-43-mediated pathology, animal models are still a widely used resource aimed at establishing pathological links between TDP-43 and ALS. These models facilitate investigations into early disease mechanisms that cannot be elucidated from post-mortem tissue, within mammalian nervous systems that are unable to be replicated in a dish. To uncover the mechanistic disease role of TDP-43 and the cell type in which misprocessed proteins act to drive pathological cascades, promotors have been used to drive varying expression levels of wild-type (WT) and mutant protein forms in murine models (Ash et al 2010, Kabashi et al 2010, Wegorzewska et al 2009, Xu et al 2011). The overexpression of wild-type TDP-43 under the control of different promotors - prion (Prp), Thy1, or calcium/calmodulin-dependent kinase (CaMKII) - results in transgenic animals with a range of phenotypes and exogenous protein expression patterns. Wild-type human TDP-43 (WT-hTDP-43) exerts toxicity in a dose-dependent manner, and induces diverging disease manifestations depending on variables such as the promoter used (Da Cruz & Cleveland 2011). Driven by Prp, a 2.5 fold WT-hTDP-43 increase in mice induces motor dysfunction, cytoplasmic protein expression, TDP-43 posttranslational modifications and abnormal molecular trafficking along neuronal processes, mimicking signatures of ALS (Stallings et al 2010, Xu et al 2011). However, in comparable studies the same promotor driving hTDP-43 expression has been shown to produce no overt phenotype, whilst a range of motor features occur in different combinations in mice expressing the protein under Thy1 or CAMKII promotors (Igaz et al 2011, Wils et al 2010). Conversely, the complete knockout of the TARDBP gene is embryonically lethal, and knockout post-natally results in metabolic defects and rapid death (Chiang et al 2010, Kraemer et al 2010, Mitchell et al 2015, Sephton et al 2010, Wu et al 2010). Establishing the effects of varying expression levels of TDP-43 has been beneficial for understanding protein functions, and it is evident these changes lead to TDP-43-mediated toxicity in the central nervous system (CNS).

Mutations in the TARDBP gene have been utilised to create models of ALS phenotypes in the presence of misprocessed TDP-43, with the majority of mutations located in the CTD of the protein (Gitcho et al 2008, Sreedharan et al 2008). The A315T mutation of TDP-43 induces autosomal dominant disease

and the accumulation of dysfunctional protein at ~3 fold overexpression as compared to endogenous levels (Gitcho et al 2008). Transgenic models with the mutation undergo the loss of layer V pyramidal neurons in the motor cortex and lower motor neurons in the spinal cord at the onset of motor deficits, recapitulating ALS clinical manifestation (Herdewyn et al 2014). The hyperactivity of somatostatin interneurons has been shown to inhibit layer V pyramidal neurons in the presence of the TDP-43^{A315T} mutation early in disease, contributing to overall excitotoxicity in the motor cortices and further recapitulating the excitability of discrete cortical networks (Zhang et al 2016). Concurrent with comprised motor abilities, TDP-43^{A315T} mice develop deficits in spatial memory and disinhibition; intriguingly, the suppression of the TDP-43 mutant in these animals for a week has the propensity to rescue motor and behavioural deficits (Ke et al 2015).

Two other key mutations of TARDBP that promote toxicity in the CNS are the M337V and Q331K mutants (Rutherford et al 2008). Both mutations provoke age-dependent, progressive axonal degeneration and neurodegeneration, gait and motor disturbances, and enhance TDP-43 splicing functions for some RNA-targets, but a loss-of-function for others (Arnold et al 2013, Bilican et al 2012, Xu et al 2011). TDP-43^{Q311K} models have been shown to develop early synaptic disturbances alongside an ALS phenotype, whereby excitatory synaptic inputs and dendritic spine densities are increased presymptomatically (Fogarty et al 2016). The most crucial finding from models of TARDBP mutations and altered protein expression in ALS is that aggregation appears to be a secondary, rather than a primary pathological mechanism (Janssens et al 2013). The vast majority of disease mechanisms identified in these models are recognisable in human cases of ALS, and each mutation induces the clinical and molecular manifestation of disease; but without widespread TDP-43 aggregation. It may well be that the aggregation of TDP-43 is a late-stage disease signature, or even an attempt by the cortex to compensate for earlier insults. Whatever may be established as the initiating pathology mediated by TDP-43, it is apparent the misprocessing of the protein is a lynch pin in the disease cascade that becomes ALS.

1.3.2 TDP-43-mediated pathology in Amyotrophic Lateral Sclerosis

The caveat of our understanding of ALS is that we have yet to definitively demonstrate whether TDP-43 misfolding induces the disease cascade or if indeed, aggregation is a result of earlier insults (Arnold et al 2013). To effectively tailor therapeutic interventions, it is essential to develop a detailed understanding of TDP-43 functions and explore the toxic effects of protein dysfunction on these roles. A diverse range of cellular stresses - including osmotic stress, endoplasmic reticulum stress, heat stress and, as aforementioned, oxidative stress - induce TDP-43 incorporation into stress granules, and the

protein directly binds to proteins associated with stress granule recruitment *in vitro* and in human tissue from ALS patients (Anderson & Kedersha 2009, Barmada et al 2014, Dewey et al 2011, Liu-Yesucevitz et al 2010, McDonald et al 2011). While the regulated aggregation of RBPs into stress granules is normally reversible, under conditions of chronic neuronal stress, stress granules may act to spread the pathological transformation of TDP-43; and intriguingly, TDP-43 within stress granules is detergent resistant (King et al 2012, Meyerowitz et al 2011).

Translocation from the nucleus, to the cytoplasm, and to association with RNA granules is a common process for RBPs, and TDP-43 has been identified as an integral protein functioning to compartmentalise and traffic mRNAs from the cell body to distal sites for localised protein translation (Batista & Monday 2016, Elvira et al 2006). Translation of mRNAs is repressed during transport until arrival at a pre-destined site, where these mRNA packages are specifically targeted to and translated at activated synapses (Steward et al 1998, Steward & Worley 2001). Within dendrites, the TDP-43^{A315T} mutant increases the size of neuronal RNA-granules and reduces movement and mobility along the axon; disrupting the arrival of key synaptic mRNAs at distal regions such as the synapse (Alami et al 2014). Taken together, these findings indicate a pathological role for TDP-43 outside of the soma and within neuronal processes.

RNA granules- along with other non-membrane-bound organelles enriched in RNA and proteins- show liquid droplet properties, such as rapid dissolution and assembly (Hyman et al 2014, Patel et al 2015). The structure of TDP-43 facilitates this process, possessing RRMs and a low complexity CTD (LCS) (Bolognesi et al 2016). The LCS drives self-assembly of RNA granules through a process that has been termed liquid-liquid phase separation (Han et al 2012, Li et al 2018, Murakami et al 2015, Patel et al 2015). ALS-linked TDP-43 mutations are known to disrupt the self-assembly or phase separation ability, with dysfunction intrinsically linked to the protein's cellular environment (Conicella et al 2016; Li et al 2018). The roles for TDP-43 as a component of various granules travelling to distal regions are indicative of functions outside of the classical nucleus-cytoplasm shuffling, aggregation-prone properties, and crucial roles for TDP-43 directly at the neuronal synapse.

1.4 TDP-43 AT THE SYNAPSE

The transport of mRNA and RNA to synapses is fundamental to the maintenance of neuronal activity and plasticity (Swanger & Bassell 2011). Specific RNAs depleted following TDP-43 knockdown are physiologically required for encoding proteins involved in synaptic activity and function

(Polymenidou et al 2011; Tollervey et al 2011). These include the subunit 2A of NMDARs; the ionotropic glutamate receptor 6 (GluA6); the calcium activated potassium channel alpha (Kcnma1); the voltage dependent calcium channel (Cacna1c); and synaptic cell-adhesion molecules neurexin 1 and 3 (Nrxn1, Nrxn3), with crosslinking immunoprecipitation sequencing (CLIP-seq) identifying glutamate transport 1 (Glt1) as an *in vivo* RNA target (Polymenidou et al 2011). In patient tissue, changes to TDP-43 binding of individual transcripts have been identified in Nrxn3, as well as the excitatory amino acid transporter-2, essential for synaptic glutamate clearance; these transcript expression changes are proportional to the binding capacity of TDP-43 (Tollervey et al 2011). TDP-43 may also be involved in directly regulating local translation at the synapse; the protein is actively relocated to the synapse following potassium chloride (KCl) stimulation of rat hippocampal neurons (Wang et al 2008). At the translational level, TDP-43 specifically targets and regulates futsch/microtubule-associated protein 1b (MAP1B), and Ras-related C3 botulinum toxin substrate 1 (Rac1) (Godena et al 2011, Majumder et al 2012). Both of these proteins are essential for the structure of synaptic compartments, suggesting TDP-43 dysregulation and misprocessing may be linked to synaptic dysfunction (Ratti & Buratti 2016).

1.4.1 TDP-43 at the presynapse

Synaptic transmission is mediated by the regulated release of neurotransmitters from the pre-synaptic compartment (Binotti et al 2016). At the pre-synapse, efficient trafficking of mRNA for local protein synthesis is vital to maintaining transmission within cortical circuitry (Alami et al 2014). Following TDP-43 silencing, pre-synaptic targeted proteins found to be differentially upregulated within primary cortical neurons include syntaxin-1A (Stx1A), vesicle-associated membrane protein 1 (VAMP1) and synaptotagmin 17 (Syt17); all essential for synaptic function and structure (Honda et al 2013). Furthermore, at disease onset whole brain protein homogenates from hTDP-43 mice display a 44% decrease in synaptophysin levels, a pre-synaptic protein often used as a marker of synaptic density (Medina et al 2014). Accordingly, TDP-43 is localised to the presynaptic mouse NMJ, concentrated at the motor nerve terminal (Godena et al 2011; Narayanan 2013).

The NMJ is a specialised cholinergic synapse that is particularly vulnerable in ALS, and facilitates signalling between muscles and nerves required for skeletal muscle function (Campanari et al 2016). Impairment of the NMJ is manifested through muscle weakness or paralysis, key features of ALS, and these symptoms have been recapitulated in a range of animal models (Ciura et al 2013, Fischer et al 2004, Magrané et al 2009). In *Drosophila,* TDP-43 mutations alter the organisation of the NMJ, correlating with reduced levels of the protein *futsch/*MAP1B-essential for microtubule assembly of the

cytoskeleton (Godena et al 2011). TDP-43^{Q331K} mutant mice display defective fusion and release of synaptic vesicles, with aberrant NMJ innervation; this occurs prior to symptom onset, indicative of a failure of the NMJ to remodel in response to activity (Chand et al 2018). Indeed, accumulating studies indicate TDP-43 may have a crucial role in maintaining the structural integrity of the NMJ and the pre-synaptic compartment and increasingly, the protein is linked to both the normal function and pathological dysfunction of the synapse (Estes et al 2013, Feiguin et al 2009, Romano et al 2014).

1.4.2 TDP-43 at the postsynapse

TDP-43 reaches the post-synaptic compartment following neuronal depolarisation, and via RNA granules (Wang et al 2008; Liu-Yesucivitz et al 2014). The protein has been shown to be vital for regulation of the Rho family of GTPases, including RhoA1, Rac1 and Cdc42, suggesting roles in actin cytoskeletal dynamics and neurite outgrowth (Koh 2006, Stankiewicz & Linseman 2014). Under normal conditions, TDP-43 inhibits Rac1 through a pathway mediated by the fragile X mental retardation protein (FMRP). TDP-43 binds to Rac1 mRNA, recruits an FMRP complex and interacts with eukaryotic translation initiation factor (eIF4E) to inhibit translational initiation of Rac1 mRNA (Majumder et al 2016). Thus, protein misprocessing induces dysregulation of Rac1 expression, inhibiting the function of a key post-synaptic protein within a structural pathway. The depletion of TDP-43 also increases levels of essential post-synaptic MAP1B and ionotropic glutamate receptor 1 (GluA1) mRNA, but only in conjunction with the depletion of FMRP (Majumder et al 2016), indicating RNA-regulatory roles as mediated by the ability of TDP-43 to bind to other proteins.

TDP-43 has been further linked to the expression levels of post-synaptic proteins involved in synaptic organisation and transmission regulation (Wang et al 2008; Majumder et al 2012; Honda et al 2013; Majumder et al 2016). The src-like adaptor protein (Slap) is differentially upregulated following TDP-43 knockdown and is recruited at the post-synaptic membrane in conjunction with NMDARs, indicating the potential for TDP-43 misprocessing to impact this key activity-dependent process (Semerdjieva et al 2013). NMDAR regulation is potentially disrupted by TDP-43 silencing, as evidenced by the alternative splicing of the CAMK2A and KCNIP genes. These genes are essential for long-term potentiation and plasticity through roles in regulating NMDARs, AMPARs and Kv4-transient potassium channels (Birnbaum et al 2004, Mathie et al 1998, Stephenson et al 2017).

Additional evidence for TDP-43 functions in synaptic receptor regulation comes from investigations into AMPA receptors. At AMPARs, the expression level of TDP-43 directly correlates with subunits GluA1 and GluA2, essential for the retention of long-term potentiation (LTP) and regulating calcium permeability (Gulino et al 2015, Isaac et al 2007, Lee et al 2003). GluA2 pre-mRNA is subject to RNA

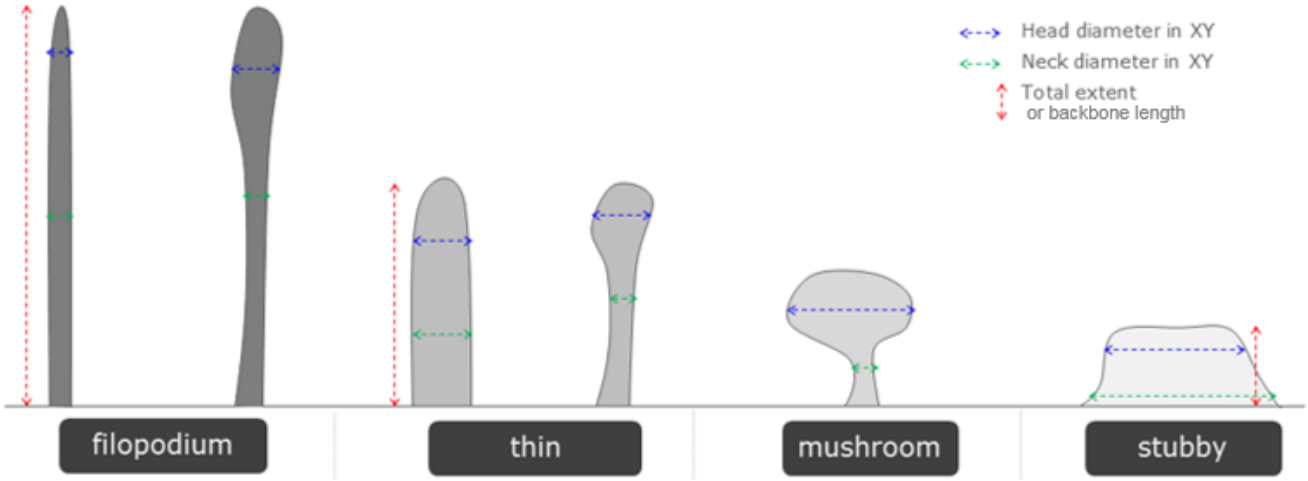
editing at the glutamine/arginine (Q/R) site, where adenosine is converted to inosine (A-to-I conversion) becoming GluA2R (Kawahara et al 2004). Failure of Q-to-R conversion results in increased excitation of neurons, and in the spinal motor neurons of sporadic ALS patients, RNA editing at this site is inefficient or abnormal (Hideyama et al 2012, Higuchi et al 2000, Kawahara et al 2004). The levels of the enzyme adenosine deaminases that act on RNA (ADAR2) have been linked to expression of the GluA2Q subunit, and in sALS, pathological TDP-43 aggregates have been identified in the spinal cord in the presence of low ADAR2 levels (Aizawa et al 2010, Hideyama et al 2012, Yamashita et al 2012). Furthermore, assays probing abnormal cleavage of TDP-43 indicate that calpain is activated through increased calcium permeability of AMPARs, thus inducing fragmentation and abnormal localisation of TDP-43 at the post-synapse (Yamashita & Kwak 2014a). These findings provide intriguing evidence for the potential of TDP-43 to induce dysfunction at the post-synapse; though it is yet to be determined whether these events may occur and what processes are initiating factors. The regulation of neuronal excitability is intimately linked the health of cells, and associated with mechanisms at the primary post-synaptic structure: the dendritic spine.

1.5 THE ROLE OF DENDRITIC SPINES

Few studies have yet to explore how changes in connectivity in ALS may be linked to the post-synaptic structure responsible for network plasticity. The majority of excitatory synapses reside at the dendritic spine as small, dynamic protrusions from the shaft of dendrites (Harris & Kater 1994, Lu & Zuo 2017). The dendritic spine contains the postsynaptic molecular machinery that enables synaptic transmission and plasticity, functionally compartmentalising intracellular chemical and electrical signalling (Bourne & Harris 2008, Nishiyama & Yasuda 2015, Sheng & Kim 2011). Dendritic spines are highly activity-dependent and exhibit a wide range of sizes and shapes within a single dendritic section, varying among neuronal type (Bosch & Hayashi 2012, Nimchinsky et al 2002). Three primary spine types are recognised; long, thin spine types are immature, transient and highly motile, the mature, strong spine types exhibit a predominantly mushroom shape, and the other stable spine type is known as a stubby spine (Alvarez & Sabatini 2007, Holtmaat et al 2005) (Figure 1.1.6). The size and morphology of the spine head is associated with the number of pre-synaptic vesicles docked there and the number of post-synaptic receptors; thus, it is also associated with the strength of synaptic currents (Arellano et al 2007, Yuste & Bonhoeffer 2001). In the post-synaptic membrane of dendritic spines, the number of AMPARs at the spine head in particular is correlated with the maturity and stability of the synapse (Matsuzaki et al 2001). AMPARs are highly dynamic and are laterally trafficked to and from the cell surface; as such, changes in AMPAR numbers at the synapse facilitate alterations to synaptic

Figure 1.1.6

Morphological subsets of dendritic spines. The function of dendritic spines is tightly linked to morphology, as indicated by the ratio head diameter (blue dashed lines) to neck diameter (green dashed lines), as well as the overall size of the structure (red dashed lines). Filopodium and thin spine types constitute immature synapses, and display a low head:neck ratio. Stubby spines are a stable spine type, whilst mushroom spines constitute the most mature dendritic spine morphology, with a large head:neck ratio.


Head diameter in XY
Neck diameter in XY
Total extent
or backbone length
filopodium
thin
mushroom
stubby

transmission (Nishimune et al 1998, Shi et al 2001). Accordingly, a reduction in post-synaptic scaffolding proteins such as PSD-95 decreases the number of receptors at the spine head as well as miniature excitatory postsynaptic current (mEPSC) amplitude (MacGillavry et al 2013, Nair et al 2013). The diffusional exchange of AMPARs between the synaptic membrane and peri-synaptic regions enables neurons to intricately regulate short-term plasticity; rapid exchange allows AMPARs to recover from desensitisation to glutamate (Heine et al 2008, Lisman & Raghavachari 2006).

The continual emergence, morphological alteration and retraction of dendritic spines in different brain regions reflect turnover and formation of synapses (Chen et al 2014, Fu & Zuo 2011, Holtmaat & Svoboda 2009). Dendritic spine dynamics are influenced spatially by cortical region and cortical layer, and the inputs discrete regions receive. While little research has looked into region-specific comparisons of dendritic spine turnover, innate functional differences suggests neuronal plasticity and circuitry mediated by dendritic spines may vary (Benavides-Piccione et al 2002, Holtmaat et al 2005, Zuo et al 2005). Activity-dependent mechanisms have repeatedly been associated with regional vulnerability in neurodegeneration (Bero et al 2011, de Haan et al 2012, Seeley et al 2009, Zhou et al 2012). The intricate relationship between dendritic spines and neuronal network activity may thus play a role in determining the regional susceptibility of neuronal populations in neurodegenerative disease. Synaptic plasticity is attributed to structural changes in the number and shape of dendritic spines, dependent on the cortical environment, with changes occurring within minutes that may endure for spans of weeks to months (Fu & Zuo 2011, Lendvai et al 2000, Yang et al 2009). Understanding fundamental dendritic spine plasticity and the factors influencing this within specific cortical regions is essential for targeting the mechanisms that may perturb synaptic function in disease states.

1.5.1 Dendritic spines in disease states

Neurodegenerative diseases feature the progressive loss of synapses and neurons in distinct anatomical networks, resulting in hallmark clinical phenotypes; yet the events initiating synaptic loss are still unknown. Pathological changes in dendritic spines at the post-synapse can be categorised as alterations in distribution and alterations of structure (Fiala et al 2002). Distribution pathology refers to increases and decreases in dendritic spine density, and to morphological changes such as altered spine size, shape and subtype; it is this measure that provides information as to the overall function of neuroplastic mechanisms within a cortical region (Penzes et al 2011). A decreased density of dendritic spines can be linked to axonal loss and deafferentation and is most evident in forms of mental retardation and epilepsy - these conditions have decreased neuronal numbers, suggesting density loss is associated with a decline in available axonal inputs and stimulation (Huttenlocher 1991, von Bohlen Und Halbach

2010, Wong & Guo 2013). Conversely, increased spine density is much less associated with disease and instead can be observed during critical developmental time periods, following environmental enrichment and in fluctuations of the oestrus cycle (Berman et al 1997, Huttenlocher & Dabholkar 1997, Munoz-Cueto et al 1990). An overabundance of dendritic spines has however been identified in Fragile-X Syndrome and in some cases of Sudden Infant Death Syndrome (SIDS), thought to be a product of defective dendritic spine pruning during development (Irwin et al 2001, Takashima et al 1994). Dendritic spine morphology on the other hand depends not only inputs received, but also on the functionality of the structure and localised protein synthesis (Fiala et al 2002, Lendvai et al 2000).

A number of possible causes have been linked to dendritic spine pathology, yet no single disease pathway has been identified. Changes in spine density can be linked to altered presynaptic inputs, compensation for the loss of afferents, autonomous neuronal functions-such as NMDAR activation, disrupted dendritic transport and aberrant protein synthesis-or due to extraneuronal factors (Coleman & Riesen 1968, Hasbani et al 2001, Keck et al 2008, Tolino et al 2012). In ALS there is an increasing focus on the effect dendritic spine aberrations have on vulnerable networks. In a mouse model of mutated ubiquilin 2 (UBQLN2) - a mutation associated with ALS- aggregation of proteins within the spine head occurs concurrently with a decrease in dendritic spine density and synaptic dysfunction (Gorrie et al 2014). Conversely, familial ALS mutations in the actin-binding protein profilin 1 have been shown to increase dendritic spine density, highlighting the complex nature of disease-mediated alterations at the post-synaptic structure (Brettle et al 2015). In ALS there is an acknowledged lengthy pre-symptomatic period, during which dysregulated cortical excitability is thought to be integral to disease onset (Joyce & Carter 2013, Menon et al 2017, van Zundert et al 2012, Vucic et al 2008). While evidence for TDP-43 mediating both physiological dendritic spine function and disease-state dysfunction accumulates, questions still remain as to whether protein dysfunction is a direct cause or consequence of dendritic spine alterations. Additionally, there is yet to be a cohesive understanding as to how and when dendritic spine alterations result in pre-symptomatic functional pathology within vulnerable cortical regions, with a need to understand fundamental changes that occur at the dendritic spine within these regions.

1.6 THESIS HYPOTHESIS AND AIMS

Early synaptic dysfunction and perturbed neuroplasticity has increasingly been implicated in the onset and progression of ALS, yet our understanding of the relationship between TDP-43 and neuroplastic mechanisms at the dendritic spine is wanting. The complexity of mechanisms occurring at the dendritic spine contributes to the lack of understanding as to normal roles, as well as how dendritic spines are affected in disease states. Roles for TDP-43 at the post-synaptic structure have been identified under normal conditions, but how protein misprocessing at the dendritic spine impacts susceptible neuronal populations in ALS has yet to be elucidated. To understand how TDP-43 may contribute to the select vulnerability of the motor system, early dysfunction at the synapse in ALS will be investigated at the dendritic spine within a mouse model of mutated TDP-43.

Aim 1: Investigate whether the synapse is dysfunctional over a disease time course in the *Thy1-YFP:prpTDP-43^{A315T}* mouse model of ALS. *Hypothesis 1: Dysfunction at the post-synaptic dendritic spine is an early disease mechanism mediated by misprocessed TDP-43, central to disease progression.*

A range of experiments support the notion of early, subtle cellular events occurring distally at the synaptic extremity, preceding neuronal death. However, investigations into the major regulators of synaptic plasticity- dendritic spines- in disease states have yet to determine whether these events are upstream of a disease cascade, or a consequence of protein pathology. The first aim of this thesis will be focused on identifying synaptic alterations at defined stages of early disease progression in a model of the ALS that recapitulates key features of disease manifestation. Mice will be probed for synaptic markers and dendritic spine alterations at a juvenile stage; presymptomatically; and at disease onset.

Aim 2: Determine whether cortical region, age and sex influence dendritic spine changes in the *Thy1-YFP* mouse model. *Hypothesis 2: Dendritic spine turnover and morphological subsets have a unique pattern of dynamics depending on cortical region, sex and age.*

To identify why the dendritic spine is vulnerable to pathological changes in disease, there needs to be a better understanding of fundamental dendritic spine dynamics. Between cortical regions, dendritic spine maturation fine-tunes specific cortical networks as a function of differences in activity-dependent mechanisms. The second aim of this thesis will be to investigate how dendritic spine dynamics change over time from developmental stages to adulthood within two distinct cortical regions of mice expressing yellow fluorescent protein. To capture the dynamic processes of dendritic spines, cranial window surgeries will be used in conjunction with 2-photon microscopy (2PLSM) to determine

alterations in spine dynamics in real time. These changes will be compared between males and females, in order to determine the role of the sex hormone oestrogen in dendritic spine regulation.

Aim 3: Investigate dendritic spine plasticity in real-time in the *Thy1-YFP:prpTDP-43^{A315T}* mouse model of ALS. *Hypothesis 3: Misprocessed TDP-43 will affect the dynamics of dendritic spine turnover and perturb neuronal plasticity.*

In ALS, distinct cortical regions are progressively impacted by the degeneration of neuronal networks. The maturation and turnover of dendritic spines is a key regulator of neuronal network plasticity and dysfunction of the structure may have vast implications for cortical circuitry in disease. To establish the potential impact of a disease linked TDP-43 mutation on the synaptic compartment, cranial window surgeries will be used in conjunction with 2PLSM to establish dendritic spine activity in the male and female motor cortices. These experiments will be undertaken at a presymptomatic time point to further build upon the timeline of synaptic dysfunction determined in Aim 2, utilising the *Thy1-YFP:prpTDP-43^{A315T}* mouse model of ALS.

2 MUTANT TDP-43 MEDIATES EARLY SYNAPTIC DYSFUNCTION

2.1 INTRODUCTION

The cytoplasmic aggregation of misprocessed TDP-43 is a key pathological feature of a range of neurodegenerative disorders and the discovery of intracellular TDP-43 inclusions in over 95% of heritable and sporadic forms of ALS implicates a shared pathological pathway for both disease forms. Molecularly, neurons depleted of TDP-43 or expressing a dominant mutation show morphological and molecular changes that indicate neuronal and synaptic dysfunction. Furthermore, being a component of transporting RNA granules provides a pathway for the protein to be directly involved in regulating synaptic strength through localised translation-the underlying mechanism of synaptic plasticity (Bliss & Collingridge 1993). Given the evidence for TDP-43 binding to RNA targets critical for synaptic function, as well as mediating dendritic spine maturation, it is entirely conceivable the protein may regulate synaptic plasticity through processes such as RNA transport, local translation and dendritic spinogenesis (Majumder et al 2012, Narayanan et al 2013, Sephton et al 2010, Wang et al 2008a, Xiao et al 2011). However, even with hints of TDP-43 functioning at the synaptic compartment, there is yet to be a cohesive understanding of what these roles encompass-let alone of how these roles may be perturbed in disease states. Potentially, in ALS the modulation of synaptic plasticity by misprocessed TDP-43 may induce synaptic deficits that in turn, are a critical turning point in the progression and onset of neurodegeneration (Bellucci et al 2012, Palop et al 2006, Sephton & Yu 2015, Sheng & Cai 2012). Dendritic spines are the major regulators of synaptic plasticity, yet it is still to be determined as to whether alterations at the postsynaptic structure are upstream of disease events, or a consequence of disease pathology. Among recent findings, a theme emerges whereby TDP-43-mediated synaptic mechanisms are significantly altered by misprocessed protein forms, potentially at early stages of the disease cascade. To understand how these insults are situated within the context of disease progression, a timeline of TDP-43-mediated synaptic dysfunction is essential.

To address these research questions, this Chapter aimed to establish a timeline of TDP-43 mediated synaptic alterations in the cortex of the TDP-43^{A315T} mouse model of ALS (Wegorzewska et al 2009). These mice express a human TDP-43 construct containing the missense mutation Ala-315-Thr (A315T) seen in familial disease (Gitcho et al 2008), and driven by the mouse prion protein promotor at 3-fold overexpression (Wegorzewska et al 2009). Previously, TDP-43^{A315T} mice died spontaneously before full disease expression due to an enteric phenotype resulting in intestinal obstruction; however, a high fat/low fibre diet abolishes sudden death, allowing a pronounced disease phenotype to develop and exhibiting a greater loss of upper and lower motor neurons (Herdewyn et al 2014). These mice are

born without a disease phenotype, and appear normal until ~3 months of age, when they first develop gait abnormalities. By 4-5 months of age, TDP-43^{A315T} mice begin losing weight and develop characteristic motor deficits, whereby they are unable to hold their bodies off of the ground but can still propel themselves forward using their limbs (Wegorzewska et al 2009). Additionally, mouse models of the A315T mutation also exhibit cognitive deficits such as impaired spatial memory acquisition and retention, increased disinhibition and hyperactivity, associated with the expression and deposition of dysfunctional TDP-43 (Ke et al 2015).

Ubiquitinated aggregates are a defining feature of TDP-43 pathology, found in the brains and spinal cords of ALS patients (Leigh et al 1991). Sufferers of ALS exhibit the degeneration of the layer v pyramidal neurons within the motor cortex- cells essential for integrating sensory cues and controlling motor output (Agosta et al 2012). Despite the universal expression of the Prp TDP-43^{A315T} transgene within all cortical layers, TDP-43^{A315T} mice only display cytoplasmic accumulation of ubiquitinated proteins in layer v cortical neurons- further demonstrating the benefits of using this mouse to investigate cortical pathology and highlighting the select vulnerability of excitatory neurons. Due to the previously observed spontaneous death of mice before full disease expression, the TDP-43^{A315T} model has been poorly utilised, and the potential for identifying early disease events in the cortex is yet to be fully explored.

The potential for synaptic dysfunction to be a key, early disease event was probed by determining alterations at the dendritic spine at defined timepoints, establishing a time-course of disease in the TDP-43^{A315T} mouse. The TDP-43^{A315T} mouse is crossed with Thy1-YFPH mice, preferentially fluorescing excitatory LV motor and sensory neurons- enabling the visualisation of regions of interest under pathological conditions. Immunohistochemistry is used to determine TDP-43 localisation and global expression of synaptic proteins. Specialised dendritic spine analysis software is used to assess changes in dendritic spine density as a whole and within morphological subsets. To elucidate the functional outcomes of any alterations, electrophysiology is used to determine the viability of the synapse. The current Chapter aimed to investigate whether the synapse is dysfunctional over a disease time-course in the *Thy1-YFP:prpTDP-43^{A315T}* mouse model of ALS, to elucidate whether this may be an early disease mechanism mediated by misprocessed TDP-43.

2.2 METHODS

2.2.1 Animals

Thy1-YFP transgenic mice [B6.Cg-Tg (Thy1-YFP-H)16Jrs/J) (Feng et al. 2000; Porrero et al. 2010)] and TDP43^{A315T} transgenic mice [B6.Cg-Tg (Prnp-TARDBPxA315T)95Balo/J (Wegorzewska et al. 2009; Herdewyn et al. 2014)] were purchased from the Jackson Laboratory and maintained on a C57BL6 background, with these strains crossed to create the novel *Thy1-YFP:prpTDP-43^{A315T}*. *Thy1-YFP* positive littermates were used as controls. These mice demonstrate an approximate 3-fold increase in expression of human TDP-43^{A315T} in comparison to endogenous TDP-43 levels, driven on the prion promoter; this drives high expression within the brain and spinal cord, as well as at lower levels in most other tissues (Wegorzewska et al. 2009). Animals were housed in ventilated cages at 20^{o}C, on a 12 hour light-dark cycle, with access to food and water *ad libitum*. All procedures were approved by the Animal Ethics Committee of the University of Tasmania and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 2013.

2.2.2 Genotyping

Mice were genotyped at the time of weaning (28 day post-natal) using a clipping from the top portion of the ear, which was removed and stored at -20°C until DNA extraction was undertaken. DNA was extracted using an Extract-N-Amp Tissue PCR tissue kit (Sigma-Aldrich, Australia) according to manufacturer's instructions and stored at -4°C. TDP-43^{A315T} PCR amplification was performed using an 11µl reaction containing 50-100g of DNA (~1µl), and the following primers (GeneWorks, Australia):1µl oIMR 8744 internal positive control forward (5'-CAA ATG TTG CTT GTC TGG TG-3'); 1µl oIMR 8745 internal positive control reverse (5'-GTC AGT CGA GTG CAC AGT TT-3'); 1µl transgene forward (5'-GGA TGA GCT GCG GGA GTT CT-3'); and 1µl transgene reverse (5' TGC CCA TCA TAC CCC AAC TG-3'). Amplification occurred under the following conditions: 94°C for 3 minutes; 94°C for 30 seconds; 60°C for 30 seconds; 72°C for 30 seconds; steps two-four repeated for 30 cycles; 72°C for 30 seconds; 4°C on infinite hold.

Thy1-YFP PCR amplification was performed using a 10µ reaction containing 50-100g of DNA (~1µl) and the following primers (GeneWorks, Australia): 1µl oIMR 7338 internal positive control forward (5'-CTA GGC CAC AGA ATT GAA AGA-3'); 1µl oIMR 7339 internal positive control reverse (5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3'); 1µl transgene forward (5'-TCT GAG TGG CAA AGG ACC TTA G-3'); and 1 µl transgene reverse (5'-TGA ACT TGT GGC CGT TTA CG-3').

Amplification occurred under the following conditions: 95°C for 1 minute; 95°C for 15 seconds; 58°C for 15 seconds; 72°C for 30 seconds; steps two-four repeated for 35 cycles; 72°C for 7 minutes; 11°C on infinite hold.

2.2.3 Tissue preparation

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3 THE DIFFERENTIAL NEUROPLASTICITY OF CORTICAL REGIONS

3.1 INTRODUCTION

The development of techniques facilitating the imaging of living structures has provided new ways to understand dendritic spine structure and function; yet there remain unknowns as to how real-time changes at synaptic compartments are influenced by factors such as cortical region, age and sex hormones. The cortical control of motor function in the mouse involves the activity of pyramidal neurons in the motor /frontal cortex, constituting a higher order processing level projecting to major subcortical motor systems, and this organisation is recapitulated within the human cortex, as demonstrated by longitudinal MRI (Bergmann et al 2016, Keller 1993, Schieber 2001)qiu(Qiu et al 2018). Understanding how the hierarchical activity of the cortex influences specific regional activity at the synapse is crucial for elucidating the mechanisms of activity-dependent vulnerability, as seen in neurodegenerative disease. The current Chapter aims to determine the biological basis for the increased susceptibility of the motor cortex to synaptic dysfunction in pathological conditions, and to better understand how to target potential for therapeutic interventions aimed at maintaining appropriate synaptic connections.

A lack of consideration has been given to the role played by sex hormones in the formation and regulation of cortical networks, both in clinical human studies and in research using animal models to investigate the synapse and disease conditions (Zárate et al 2017). A range of pathologies have a clear sex differences in their prevalence, including ALS, yet the precise roles played by hormonal cues in determining disease dimorphism remain to be clarified (Arevalo et al 2015). Women experience ALS onset significantly later in life than men- corresponding to the post-menopausal period, during declining oestrogen levels (Curtis et al 2017, McCombe & Henderson 2010). Furthermore, while incidence rates occur at a ratio of 3:2 between males and females overall, this diminishes to a ratio of 1:1 with advancing age and decreased oestrogen levels in females (Blasco et al 2012, Manjaly et al 2010, McCombe & Henderson 2010). Gonadal hormone fluctuations during the oestrous cycle have well-established impacts on a range of cognitive functions in the female brain (Alexander et al 2018, Srivastava et al 2008). Delivery of estradiol- an estrogen agonist- rapidly improves discrimination learning in female mice, and within the hippocampus has been shown to dramatically and rapidly increase dendritic spine and synaptic density (Murakami et al 2006, Spencer et al 2008, Srivastava et al 2013).

Dendritic spines are also influenced by the brain region in which they reside and the stage of development the cortex is undergoing. Regional dendritic variation is extensive, with crucial implications for cortical processing, as a larger dendritic field is associated with increased spine numbers and input sampling across cortical systems (Elston & Rosa 1998, Elston et al 1996, Oga et al 2017). Spines within the frontal cortex undergo pruning during adolescence and developmental periods across a variety of species, including humans and mice (Johnson et al 2016, Petanjek et al 2011). In layer V pyramidal neurons of the frontal cortex, these changes are also mediated by pubertal development, with sex-specific changes identified in rats (Drzewiecki et al 2016). The development of stable dendritic spines and synaptic connections can also be mediated by specific cortical regions through network-wide activity- though there has yet to be comprehensive comparisons of inter-regional dendritic spine dynamics (Antonini et al 1999). Intriguingly, cortical regions involved in early stages of processing exhibit less complex dendritic spine turnover than those involved in later processing stages, reflecting spine-dense neurons at higher association levels integrating a greater range of synaptic input than those at lower processing levels (Jacobs et al 2001).

Classic studies demonstrate time periods of heightened neuronal plasticity- critical periods in which the development of the nervous system is able to be rapidly modulated by experience (Hubel et al 1977, Lichtman & Colman 2000, Shatz & Stryker 1978). However, the structural bases and dynamics underlying the transition from plasticity to stability are still poorly understood. Dendritic spines are widely accepted as specialised, distinct compartments, and their morphology is optimised for excitatory transmission and the formation of functional brain circuitry (Bloodgood & Sabatini 2007, Maiti et al 2015, Sala & Segal 2014). The dimensions of the spine head and neck dictate function, including the abundance of neurotransmitters; small molecule diffusion; independent regulation of biochemical and electrical machinery; and calcium compartmentalisation (Harris & Kater 1994). As the morphogenesis of dendritic spines that supports these processes is impaired in a range of disorders, understanding the factors influencing synaptic plasticity is critical for elucidating dysfunction in the face of neurodegeneration.

The ability to monitor neuronal connectivity in real time has provided new understanding of the mechanisms underlying the development of neuronal circuitry and connections- and why these networks may be primed to fail in neurodegenerative states (Okabe 2013). Two-photon laser scanning microscopy (2PLSM) facilitates the monitoring of brain structures such as dendritic spines *in vivo,* capturing changes in neuronal connectivity mediated by cognitive functions, as well as in pathological states (Yang et al 2010b). To repeatedly and reliably image the same region of interest over time, open-

skull windows facilitate optimised resolution and comparable spine turnover between groups when undergoing standardised procedures, and further protocol optimisation includes using immune suppressants such as a dexamethasone, to reduce glial activation and promote physiological conditions for spine turnover (Keck et al 2008, Park et al 2015).

To address gaps in the knowledge of fundamental dendritic spine dynamics within specific cortical regions, the second aim of this thesis was to determine whether cortical region, age and sex influence dendritic spine changes in the *Thy1-YFP* mouse. The *Thy1-YFP* mouse selectively expresses yellow fluorescent protein within layer V pyramidal neurons of motor and sensory regions, facilitating visualisation of the basal and apical dendrites of the primary motor and somatosensory cortices within a high density of cells, increasing the statistical power of analyses by providing a larger sample size for quantification (Feng et al 2000). This enables capturing the neuronal population susceptible to disease insult in ALS, to identify specific changes occurring under normal conditions that may perturbed in disease. Cranial window surgeries in conjunction with 2PLSM provide real-time quantification of dendritic spine changes over three imaging sessions within regions of interest, identifying the same dendritic process with each subsequent round. Neurolucida™ software is used to asses changes in morphology and spine turnover as a measure of baseline gain and loss rates. The experimental paradigm will be used to answer three key research questions, 1) being do sex hormones influence neuroplasticity at the dendritic spine; 2) are there regional differences in dendritic spine changes; and 3) are these specific dendritic spine changes altered over time, from adolescence to adulthood? These questions will be used to form a fundamental basis for dendritic spine changes that may underlie the select vulnerability of discrete cortical regions to neurodegenerative disease and early synaptic dysfunction.

3.2 METHODS

3.2.1 Animals

Experimental procedures utilised both male and female mice and were approved by the Animal Ethics Committee of the University of Tasmania, performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Animals were housed in ventilated cages at 20°C on a 12 hour light-dark cycle, with access to food and water *ad libitum*. *Thy1-YFP* transgenic mice [B6.Cg-Tg (Thy1-YFP-H) 16Jrs/J) (Feng et al 2000, Porrero et al 2010)] were purchased from the Jackson Laboratory and maintained on a C57BL6 background. *Thy1-YFPH* mice express high levels of yellow fluorescent protein in the motor and sensory neurons of cortical layer V (LV) (Feng et al 2000; Porrero et al 2010).

3.2.2 Genotyping

Mice were genotyped at the time of weaning (28 day post-natal) using a clipping from the top portion of the ear, which was removed and stored at -20°C until DNA extraction was undertaken. DNA was extracted using an Extract-N-Amp Tissue PCR tissue kit (Sigma-Aldrich, Australia) according to manufacturer's instructions and stored at -4°C.

Thy1-YFP PCR amplification was performed using a 10 µl reaction containing 50-100g of DNA (~1µl) and the following primers (GeneWorks, Australia): 1 µl oIMR 7338 internal positive control forward (5'-CTA GGC CAC AGA ATT GAA AGA-3'); 1 µl oIMR 7339 internal positive control reverse (5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3'); 1 µl transgene forward (5'-TCT GAG TGG CAA AGG ACC TTA G-3'); and 1 µl transgene reverse (5'-TGA ACT TGT GGC CGT TTA CG-3'). Amplification occurred under the following conditions: 95°C for 1 minute; 95°C for 15 seconds; 58°C for 15 seconds; 72°C for 30 seconds; steps two-four repeated for 35 cycles; 72°C for 7 minutes; 11°C on infinite hold.

3.2.3 Oestrus cycle tracking

In mice, the oestrus cycle is divided into 4 stages (proestrus, oestrus, metestrus and diestrus) and repeats every 4-5 days unless interrupted by pregnancy, pseudopregnancy or anestrus (Byers et al 2012) (Figure 3.2.1). The oestrus stage in which female mice underwent 2PLSM was assessed in two steps; a preliminary visual observation of the vaginal opening of each mouse, and verification by vaginal cytology. To evaluate oestrus cycle stage visually, mice were restrained and vaginal opening evaluated based on standard criteria (Byers et al 2012, Champlin et al 1973). (Figure 3.2.2 Ai-iv). A

Figure 3.2.1

Schematic of the relative time period and proportion of each cell type present during the four stages of the oestrus cycle, with the transition from each stage delineated. The size of each quadrant is an approximate length of each oestrus stage, with the total cycle taking 4-6 days. (A) Leukocytes are the predominant cell type during diestrus; both nucleated and cornified epithileal cells during proestrus and metestrus; and predominantly cornified epithileal cells during estrus. (B) Oestrogen levels peak during estrus for 24-48 hours, before lowering for 24 hours during metestrus. Oestrogen hits baseline levels during diestrus for ~48-72 hours, before rising for 14 hours during proestrus. Adapted from Byers et al 2012.

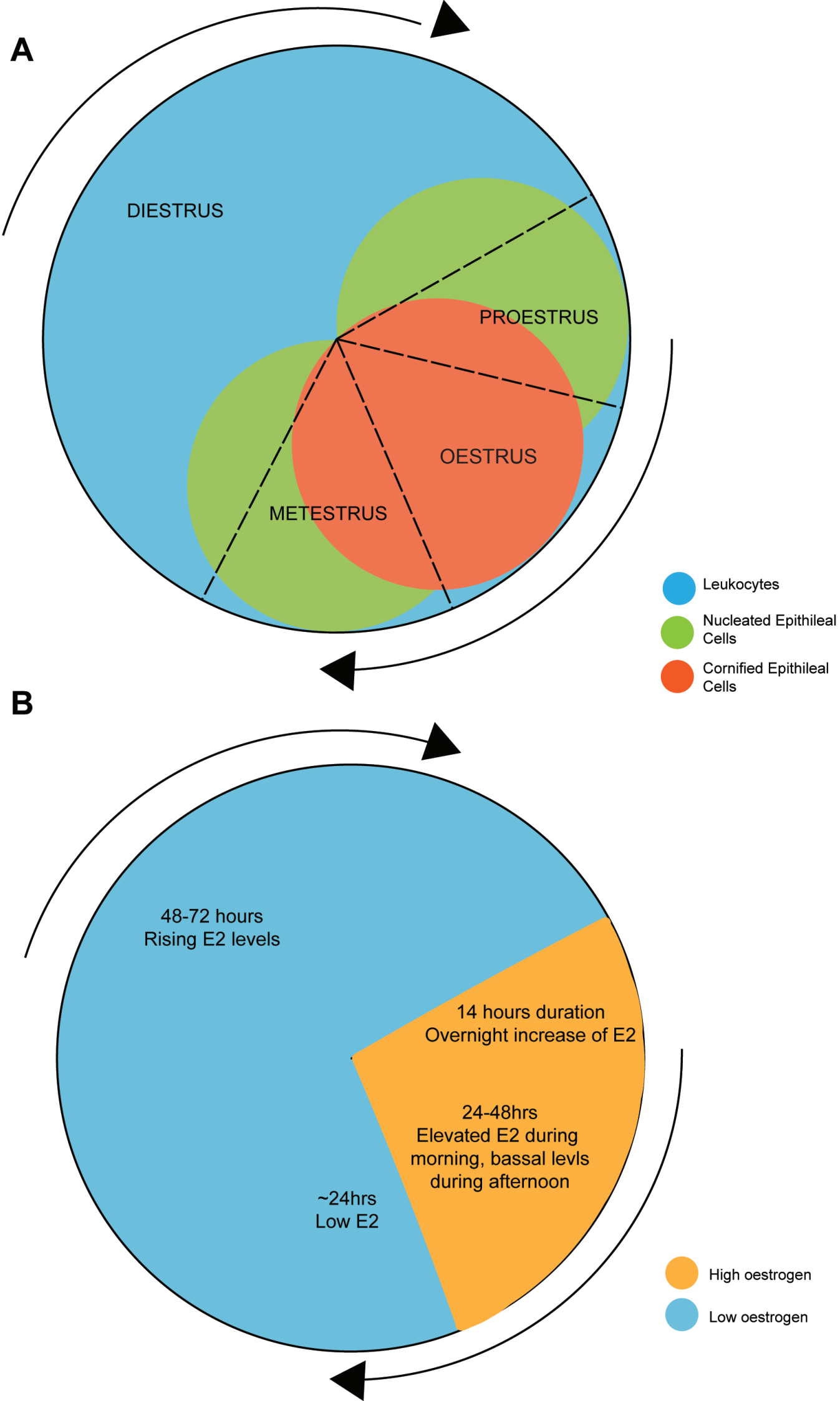
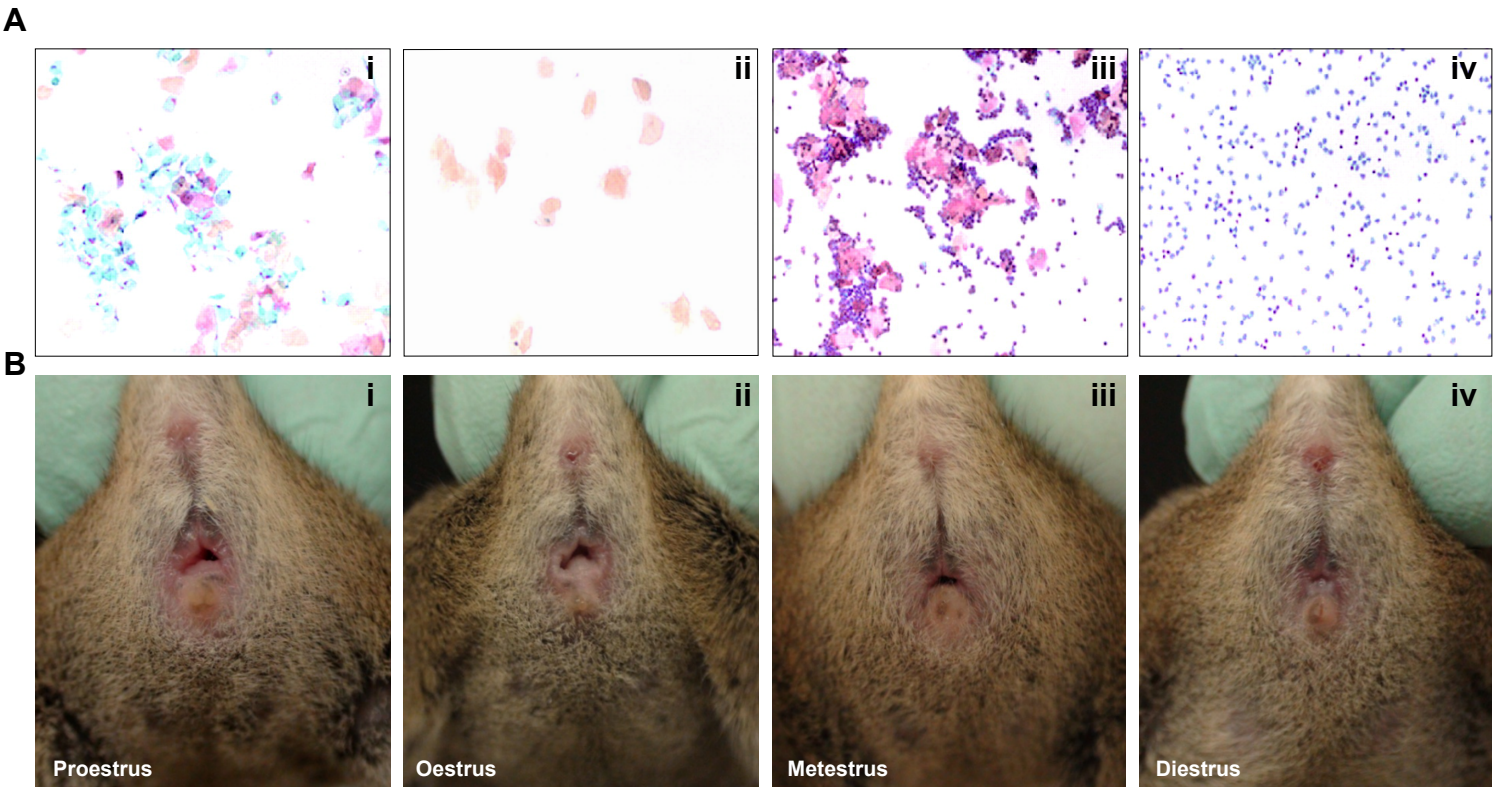

A
DIESTRUS
PROESTRUS
OESTRUS
METESTRUS
Leukocytes
Nucleated Epithileal Cells
Cornified Epithileal Cells
B
48-72 hours
Rising E2 levels
14 hours duration
Overnight increase of E2
24-48hrs
Elevated E2 during morning, bassal levls during afternoon
~24hrs
Low E2
High oestrogen
Low oestrogen

Figure 3.2.2

Representative images of the four stages of oestrus identified by vaginal cytology (A) and visual appearance (B) (Byers et al 2012; McLean et al 2012). (A) Three cell types were identified after collecting a vaginal swab, and cells transferred to a dry glass slide to be stained with toluidine blue. The stage of the oestrus cycle was determined based on the presence or absence of leukocytes, cornified epithelial and nucleated epithelial cells. Proestrus is determined by the primary presence of nucleated epithelial cells (Ai, Bi); estrus determined by the primary presence of cornified epithelial cells (Aii, Bii); metestrus determined by the combined presence of leukocytes, nucleated epithelial cells and cornified epithelial cells (Aiii, Ciii); and diestrus determined by the primary presence of leukocytes (Aiv).


A
i
ii
iii
iv
B
i
ii
iii
iv
Proestrus
Oestrus
Metestrus
Diestrus

vaginal swab was collected using a cotton tipped swab, wetted with room temperature saline. The swab was inserted into the opening of the vagina of the restrained mouse and rolled against the vaginal wall. Cells were transferred to a dry glass slide (Livingstone, Australia) by rolling the swab across the slide. Slides were then air-dried and stained with toluidine blue (0.l25% toluidine blue in 0.l25% acetic acid) (Sigma Aldrich, Australia) for 10 minutes. The slides were then rinsed with water, overlaid with a cover-slip using fluorescent PermaFluor™ mounting medium (Thermo-Fisher Scientific, Australia) and viewed immediately at 200x magnification under bright-field illumination using an Olympus CKX31 Inverted Microscope (Olympus Australia Pty. Ltd., Australia). The stage of oestrus cycle was determined based on the presence or absence of leukocytes, cornified epithelial, and nucleated epithelial cells (Felicio et al 1984) (Figure 3.2.2 Bi-iv).

3.2.4 Tissue preparation

For tissue collection mice were terminally anaesthetised with an overdose of sodium pentobarbital, and transcardially perfused with 4% (w/v) paraformaldehyde (PFA) in 0.01 M phosphate-buffered saline (PBS) at postnatal day (P) 30 (n = 5), P60 (n = 5), and P90 (n = 5). Dissected cortical tissue was postfixed overnight in 4% PFA and stored at 4°C in 0.01 M PBS containing 0.1% (w/v) sodium azide (Sigma Aldrich, Australia). The brain was cut at Bregma – 4.00mm, and the anterior portion embedded in 5% (w/v) molecular grade agarose (Bioline Australia Pty Ltd, Australia) dissolved in 0.01 M PBS. Coronal vibratome sections (20µm) were generated using a Leica VT1000S vibratome (Biosystems Australia Pty Ltd, Australia) and collected onto slides (Livingstone, Australia). 20 µm sections were processed immediately for immunohistochemistry.

3.2.5 Immunolabelling

For cortical analyses, the primary motor and somatosensory cortices were identified by anatomical landmarks referring to the appearance of the lateral ventricles, the shape of the third ventricle and the appearance of the anterior commissure and corpus callosum (Paxinos & Franklin 2012). For dendritic spine density analysis, sections on slides (20 µm) were exposed to a primary antibody solution containing 0.3% triton-X-100 (Sigma Aldrich, Australia) and anti-GFP (1:1000), which detects endogenous YFP for 24 hours at room temperature and agitated. Sections were then washed three times in PBS and incubated with an Alexa Fluor® conjugated secondary antibody (Thermo-Fisher Scientific, Australia) for 2 hours at room temperature. Sections were washed three times with PBS for 10 minutes prior to drying in the dark. Slides were mounted in fluorescent mounting medium (Thermo-Fisher Scientific, Australia). No primary controls were included in immunohistochemical experiments.

3.2.6 Confocal microscopy

Immunofluorescence was captured using a Zeiss LSM 510 Duo-scan confocal microscope (Carl Zeiss Microscopy, Germany) running ZEN software and equipped with Ar488 and HeNe543 lasers. For cell body analysis in the motor and somatosensory cortices, an EC Plan Neofluar 10x/0.3 air objective (Carl Zeiss Microscopy, Germany) was used to acquire z-plane images with 0.7 µm intervals through 12 µm of the tissue depth. Eight sections were imaged per cortical region, per mouse. To image the basal and apical dendrites of layer V pyramidal neurons, a Plan-Apochromat 63x/1.4 oil objective (Nikon, USA) was used to acquire z-plane images with 0.9 µm intervals through 18 µm of tissue. Four images were collected from eight sections per cortical region per time point (960 stacks in total).

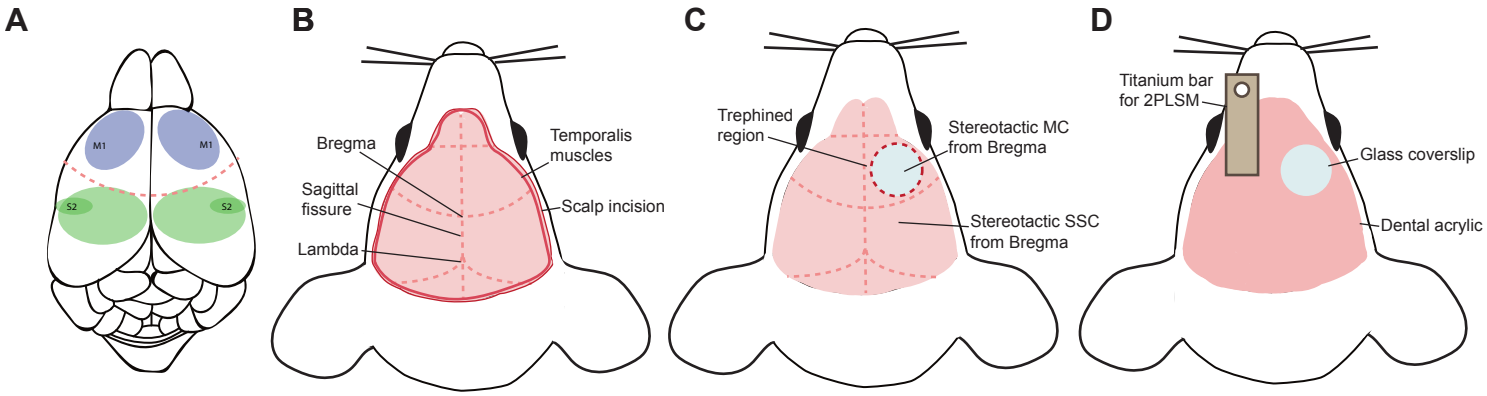
3.2.7 Cranial window surgeries

An open-skull cranial window was surgically implanted over either the motor or somatosensory cortices of mice at P50, following the protocol previously described by Holtmaat et al. (2009) (Figure 3.2.3). This timeline enables 10 days of recovery prior to imaging at P60, whereupon ~80% of cranial windows were successful. Experimental mice underwent isoflurane (Henry Schein®, USA) induction (4%, ~0.5 litre min^{-1} O_2), were given a sub-cutaneous (s.c.) injection of the long-acting general analgesic Temgesic (buprenorphine 300mg/ml dosed at 0.1mg/kg) (Ilium, Troy Laboratories, Australia) and heads shaved to prevent foreign particles entering the surgical site. Mice were then placed into a stereotaxic frame and isoflurane maintained at 1.5-2%, ~0.5 litre min^{-1} O_2. The cranium was cleaned with three rotations of a cotton swab with the disinfectant Microshield® chlorhexidine (Schulke Pty. Ltd., Australia) and after drying, swabbed again with ethanol (Sigma Aldrich, Australia). Viscotears® liquid eye gel (Novartis Pharmaceuticals, Australia) was applied and the mouse injected s.c. with the local analgesic bupivacaine (bupivacaine hydrochloride 0.5% dosed at 0.025%) (Pfizer Ltd., Australia) and the anti-inflammatory Metacam (meloxicam 5 mg/ml dosed at 1 mg/kg) (Ilium, Troy Laboratories, Australia).

Using scissors, a circular flap of skin (~1 cm^2) was removed from the skull, and the periosteum removed by gently scraping the skull with sharpened forceps. Vetbond™ (cyanoacrylate 3M™, USA) tissue adhesive was applied to the *temporalis* muscle and wound margins to prevent seepage of serosanguinous fluid, sparing the trepanation area. The cortical regions of interest were identified according to bregma, in order to accurately locate the correct coordinates for the motor cortex (-1.0 mm posterior from bregma, -0.8 mm lateral) and the somatosensory cortex (1.5 mm from bregma, 3.0 mm lateral) (Figure 3.2.3 A, B). A circular groove was drilled around the area of interest (~3 mm in diameter) and cortex buffer (125 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM $CaCl_2$,

Figure 3.2.3

Schematic of the cranial window procedure and identification of regions of interest. (A) The motor and somatosensory cortices are identified according to coordinates from Bregma. (B) A circular flap of skin is removed to reveal the cranium, the sutures of the cranium and the temporalis muscle. (C) A 3mm bone island is removed from the trabecular bone over the region of interest using a titanium drill. (D) A 5mm glass coverslip is placed over the exposed dura membrane, and the cranium, wound margins and the lip of the coverslip sealed using dental cement. A titanium steel bar with screws holes is embedded to facilitate fastening under the 2-photon laser scanning microscope. (E) Surgeries were undertaken at post-natal day 50 (P50) and 10 days of post-surgical monitoring and oestrus cycle tracking elapsed prior to 2PLSM at P60. At P60, imaging sessions occurred once every 3 days, ~24 hours apart.



E

P50 Post-surgical monitoring, oestrus cycle tracking P60

Cranial window insertion (MC or SSC)

3 x imaging sessions, 24 hours apart

2 mM $MgSO_4$) regularly applied to avoid heating. A bone island was left intact, dampened with buffer and sharp angle-tipped forceps inserted into the trabecular bone exposed on the side of the groove to expose the dura. Dexapent (dexamethasone sodium phosphate 5 mg/ml dosed at 4 mg/ml) (Ilium, Troy Laboratories, Australia) was topically applied to the surface of the exposed dura to act as an immunomodulator and prevent regrowth of neomembrane (Figure 3.2.3 C). A circular coverglass (4 mm diameter, #1 thickness) was placed over the dura flush with the skull and Loctite® adhesive (Henkel Adhesives, Australia) applied around the window edges to prevent seepage under the coverslip. The cranial window was sealed with dental cement (Paladur, Kulzer Australia Pty. Ltd., Australia), covering the exposed cranium, the wound margins and the lip of the coverglass. A titanium steel bar (Central Science Laboratory, University of Tasmania, Australia) with screw holes was embedded in the dental acrylic over the intact hemisphere for animal stabilisation during imaging sessions (Figure 3.2.3 D).

Post-surgery, the mouse received a 1 ml s.c. injection of saline and was placed back into the home cage on a heat pad for ~30 minutes of recovery. The mouse was considered fully recovered after observation of grooming, eating and normal movement and reflexes. Monitoring was undertaken twice a day for 4 days, then once a day for 6 days, prior to the first imaging round (Figure 3.2.3 E).

3.2.8 Two-photon scanning laser microscopy

To image dendritic spine dynamics in real time, a 2PLSM (Scientifica, UK) equipped with a MaiTai laser (Spectra-Physics, NewSpec Pty. Ltd., Australia) and running custom MATLAB® software (MathWorks, Australia) was used over 3 consecutive imaging sessions, 24 hours apart to image the motor and somatosensory cortices. The MaiTai laser was run at 910 nm by a 10 W solid state laser (Spectra-Physics, Australia). The mouse was anaesthetised following an intraperitoneal (i.p.) injection of a 12:1 mixture of Ketamine (ketamil 100mg/ml) (Ilium, Troy Laboratories, Australia) and Xylazine (xylazil 20mg/ml) (Ilium, Troy Laboratories, Australia) at a recommended dosage of 5 µl of working solution per gram (0.12 mg/g ketamine to 0.01 mg/g xylazine, approximate dose of 100 µl for a 20 g mouse), which wore off after 45-60 minutes. Once no reflexes were detected, mice were placed on a heat-pad maintained at 37°C under the microscope, with head immobilised by screwing the titanium bar of the cap to a stable, adjustable arm and eye gel applied.

An EC Plan-Neofluar 10x/0.3 air objective (Nikon, USA) was used to image the vasculature of a region of interest (ROI), illuminated by white light, and captured using XCAP Image Processing Software (EPIX Incorporated, USA) (Figure 3.2.4 Ai). Once a region of interest was identified, the z-plane coordinate was saved and the objective changed to a W Plan-Apo 40x/1.0 water immersion objective

(Nikon, USA). The same z-plane coordinate was initiated and a higher-powered region of interest captured to find the same position during consecutive imaging sessions (Figure 3.2.4 Aii). The MaiTai laser was used to capture blue/green emissions (Channel 1 PMT 550-565 nm) at 910 nm/1700 mW of power at a maximum percentage of 25%, equivalent to 70 mW (Mainen et al 1999). The apical tuft dendrites of LV neurons were captured in Z-stacked images (20 µm) of cortical layers II-III (slices 1 µm apart) at a minimum depth of 210 µm from the cranial surface to a maximum depth of 350 µm (Figure 3.2.4 B). Landmark dendrites were identified during the initial imaging session, and returned to in the following sessions in order to capture the same region of interest and identify the same dendritic spines on a given dendritic segment (Figure 3.2.4 C). The xyz-coordinates of every ROI were stored relative to the vasculature pattern initially captured. To verify all structures were detected, excitation was increased to ensure no additional spines were revealed and signal fluorescence intensity was different from noise. Imaging sessions lasted for ~30 minutes p/mouse and during imaging the respiratory rate of the mouse was monitored at 10 minute intervals. Post-imaging the mouse was injected with 1 ml of saline s.c. and placed back into the transfer cage on a heat pad for recovery, until fully ambulant.

3.2.9 Quantification of dendritic spine density

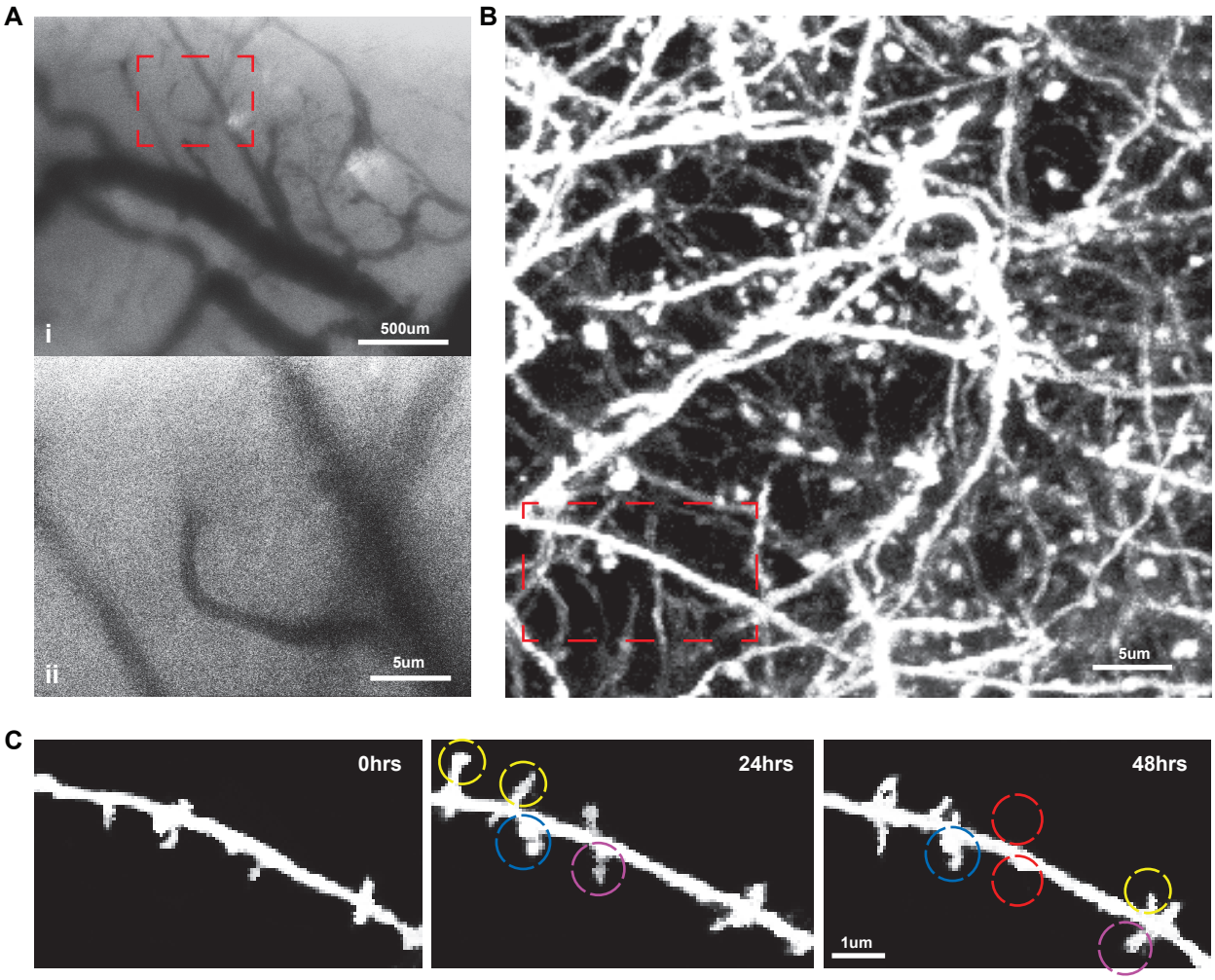
For dendritic spine density quantitation, Z-stack images of the basal and apical dendrites within the motor and somatosensory cortices were analysed using Neurolucida™ software (MBF Bioscience, USA). Dendritic compartments were located using the location of the processes; apical dendrites are found extending throughout layers II/III and IV, whilst basal dendrites are located within layer V. Image files were renamed and analysed while completely blinded; dendrites were traced through stacks with spines marked, and images then exported to Neurolucida™ Explorer 11 (MBF Bioscience, USA) for spine quantitation. Branched structure analysis was used to analyse the number of dendritic spines per µm and density of spine morphologies. Spines were classified as mature or immature according to neck length and spine head size, referring to established functional parameters (Harris et al 1992, Holtmaat et al 2009). Approximately 10 dendrites from each dendritic compartment per animal were quantified, per group- Mature spines are those with a larger head to neck ratio, and consist of stable, stronger synaptic connections; conversely, immature spines are those with less distinction between the spine head and neck, and consist of transient populations (Holtmaat et al 2009) (Figure 2.2.2 A-D).

3.2.10 Quantification of dendritic spine turnover

Z-stacked images were taken in real time of LV apical tuft dendrites residing in layers II/III of the motor and somatosensory cortices at 3 times points, 24 hours apart (Figure 3.2.5 A, B). To quantify

Figure 3.2.4

(A) Representative images illustrate the vasculature of the surface of the brain, used to locate the same region of interest for subsequent imaging rounds at 10x magnification (Ai, scale bar = 500μm) and 40x magnification (Aii, scale bar = 5μm). (B) Representative z-stacked image captured in real time of YFP-positive layer V apical tuft dendrites residing in layer II-III of the cortex. Scale bar = 5μm (C) Dendritic segments were selected for quantification based on clarity and evident spine morphologies. Dendritic spines were annotated over three consecutive imaging rounds using a 2-photon laser scanning microscope as gained (yellow) lost (red), morphological matured (purple) and stable (blue). Scale bar = 1μm


A
i
500um
ii
5um
B
5um
C
0hrs
24hrs
48hrs
1um

the turnover of dendritic spines, images for a given segment of dendrite from each round were opened in Neurolucida™ and spines annotated along the segment as being morphologically thin, stubby or mushroom shaped. The analysed segments were uploaded into Neurolucida Explorer™ and density quantified to validate representation of a given cohort. For each animal, ~2 dendrites were analysed for each cohort; P60 MC males (369 spines), baseline oestrus (406 spines) and high oestrus (350 spines); SSC males (449 spines), baseline oestrus (360 spines) and high oestrus (390 spines); P90 MC males (382 spines), baseline oestrus (293 spines) and high oestrus (404 spines); and P90 SSC males (545 spines), baseline oestrus (422 spines) and high oestrus (578 spines). Spines were only measured if they clearly emanated laterally from the dendritic shaft-those protruding below or above the dendrite could not be reliably quantified in the axial dimension. Spines for each round were quantified in comparison to the preceding round as being gained, lost or exhibiting a morphological change-with morphological subtypes being classed as mature (mushroom and stubby morphologies) and immature (thin morphology). Spines were considered lost if they disappeared between imaging rounds; gained if they newly protruded between rounds; and considered to exhibit morphological changes if the head to neck ratio was clearly altered between rounds (Figure 3.2.5 C). The baseline turnover gain and loss rates of spine subtypes was calculated as $TR = N_t/N_l$ or N_g, where TR is the turnover rate; N_t is the total number of spines for a single mouse; N_l is the number of spines lost; and N_g is the number of spines gained, as a ratio from the total number of dendritic spines and dendritic spine changes. This formula was used to calculate the total gain and loss rate of all spines for a single mouse, as well as the specific gain and loss rates of mature, and of immature spines-where N_lm and N_gm is equal to the number of lost and gained mature spines, and N_li and N_gi is equal to the number of lost and gained immature spines (Attardo et al 2015, Frank et al 2018).

3.2.11 Statistical analysis

Student's t-tests, one-way and two-way analysis of variance (ANOVAs) were performed in GraphPad (USA) Prism. Post-hoc comparisons were performed using Bonferroni's correction for multiple comparisons. A p-value of <0.05 was considered significant.

3.3 RESULTS

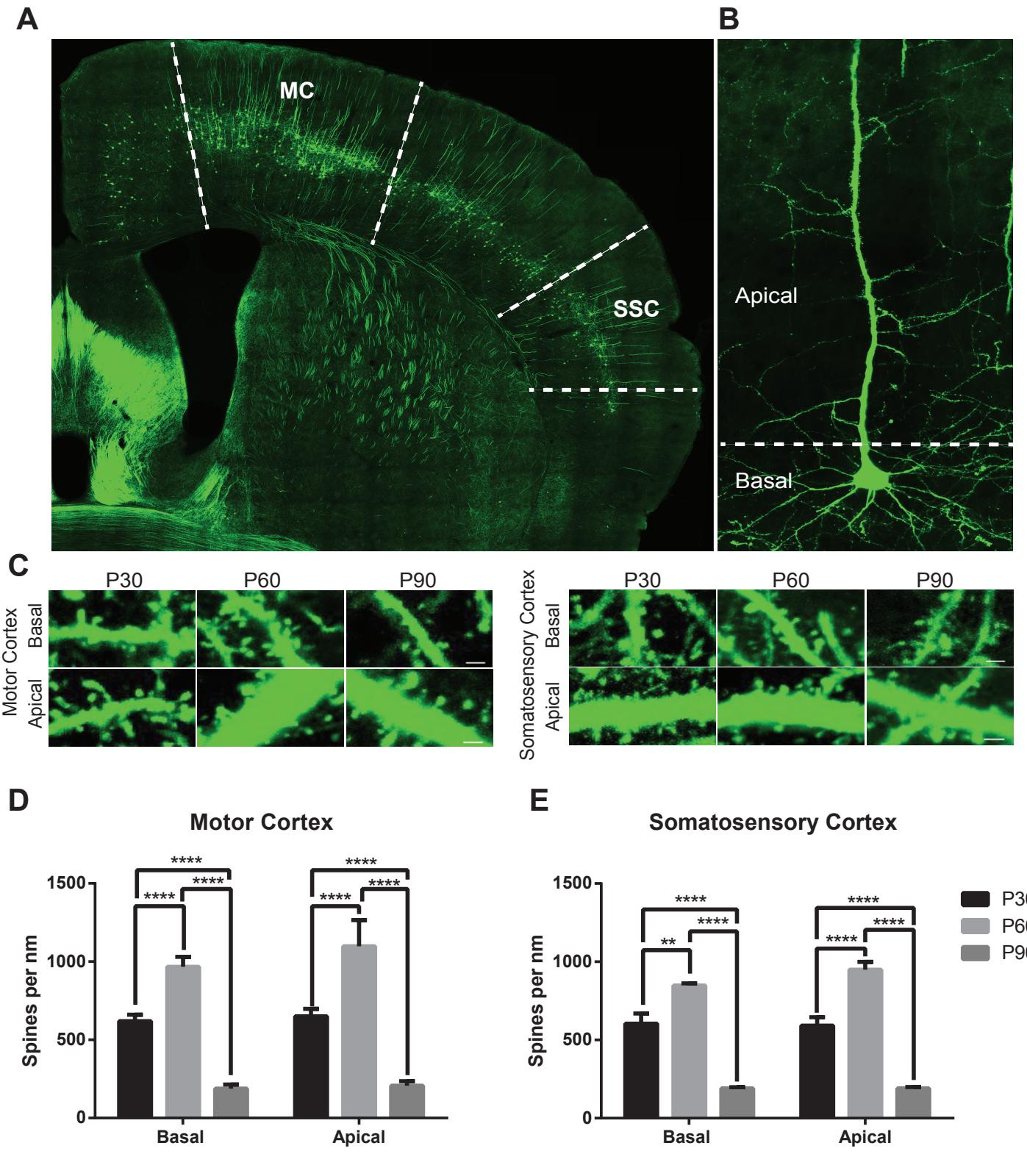
3.3.1 Dendritic spine density peaks at P60 in the *Thy1-YFP* mouse cortex

Pyramidal neurons are structurally typified by a skirt of basal dendrites and a single vertical apical dendrite emerging from the soma (Bianchi et al 2013). Generally, apical dendrites extend throughout cortical layers (Major et al 2013); in contrast, basal dendrites make localised intralayer synaptic connections (Nevian et al 2007). The previous Chapter of this thesis identified specific changes in dendritic spine density at adolescent P60 in a mouse model of ALS; to establish the overall density of dendritic spines at this time point under normal conditions across basal and apical dendritic compartments, spine density was also investigated using the *Thy1-YFP* mouse (Figure 3.3.1 A) at post-natal day 30 (P30, juvenile mouse), P60 (young adult) and P90 (adult). The *Thy1-YFP* mouse expresses YFP in layer V pyramidal neurons (Feng et al 2000) enabling the basal and apical dendrites of the primary motor and somatosensory cortices (Figure 3.3.1 B) to be clearly identified at all time points (Figure 3.3.1 C). Basal and apical dendritic spine density was quantified (spines per millimetre) in layer V of the motor cortex (MC) and the somatosensory cortex (SSC).

Within the motor cortex, the dendritic spine density of both basal and apical compartments were found to be significantly ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) increased between P30 and P60 (619.88 ± 91.02 p/nm P30 MC basal, 966.64 ± 63.44 p/nm P60 MC basal) (650.48 ± 107.52 p/nm P30 MC apical, 1098.16 ± 167.81 p/nm P60 MC apical) (Figure 3.1 D). By P90, dendritic spine density was significantly ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) reduced compared to P60 across both dendritic compartments within the motor cortex (188.50 ± 25.68 p/nm P90 MC basal) (206.34 ± 29.88 p/nm P90 MC apical) (Figure 3.3.1 D). A similar pattern emerges over time within the somatosensory cortex, whereby there was a significant ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) increase in dendritic spine density between P30 to P60 (604.52 ± 144.05 p/nm P30 SSC basal, 848.30 ± 29.13 p/nm P60 SSC basal) (592.26 ± 120.71 p/nm P30 SSC apical, 948.86 ± 111.69 p/nm P60 SSC apical), before significantly decreasing by P90 (190.25 ± 17.16 p/nm P90 SSC basal) (190.43 ± 18.69 p/nm P90 SSC apical) (Figure 3.3.1 E). Basal and apical dendritic spine density was also significantly reduced at P90 in comparison to P30 in both cortical regions. Increases in spine density are thought to reflect the capacity of a network to undergo heightened neuroplasticity during periods of development and learning (Nakamura et al 1999; Hensch et al 2005; Alvarez et al 2007). These results demonstrate that the adolescent P60 time point- where synaptic dysfunction is first observed in the *TDP-43^{A315T}* mouse- is

Figure 3.3.1

Time course of dendritic spine density in the *Thy1-YFP* cortex. (A) Whole brain image of the *Thy1-YFP* mouse, with the motor and somatosensory cortices outlined in white. (B) A YFP-positive pyramidal neuron, demonstrating the position and morphology of basal and apical dendrites. (C) Representative images show basal and apical dendritic spine density over a time course at 30 days, 60 days and 90 days (P30, P60, P90) in the motor and somatosensory cortices of *Thy1-YFP* mice. (D, E) The graphs show the quantification of spine density in the basal and apical dendrites of the motor and somatosensory cortices from $n = 5$ mice per time point. Bars show mean ± SEM. $*p = <0.05$. Scale bar (C) = 10μm, (E) = 6μm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P60
P90

a period of heightened dendritic spine density under normal conditions within the *Thy1-YFP* cortex, and that this is consistent across dendritic compartments and cortical regions.

3.3.2 Dendritic spine of the male *Thy1-YFP* motor cortex are highly dynamic at P60

To further establish the changes occurring at the dendritic spine in real time at the P60 time point, the current chapter employed 2PLSM in conjunction with cranial window surgeries to investigate dendritic spine changes occurring in the maturing cortex, during a time point where synaptic connections are being alternately strengthened and pruned (Boivin et al 2018). Individual apical tuft dendrites of layer V pyramidal neurons were followed over three consecutive days, in the motor (MC) and somatosensory (SSC) cortices of *Thy1-YFP* male (M) (n = 6) and female (F) mice (n = 8 p/group). Snapshots of dendritic spines were taken at each imaging round for comparison of dendritic spines over the three rounds. To establish the overall spine activity within different cortical regions between the sexes, the total proportion of stable and dynamic spines was quantified within each cohort. Dynamic populations were classified as mature or immature spine types, with a new spine considered 'gained' and the absence of a spine in a subsequent imaging round considered 'lost' (Figure 3.3.2).

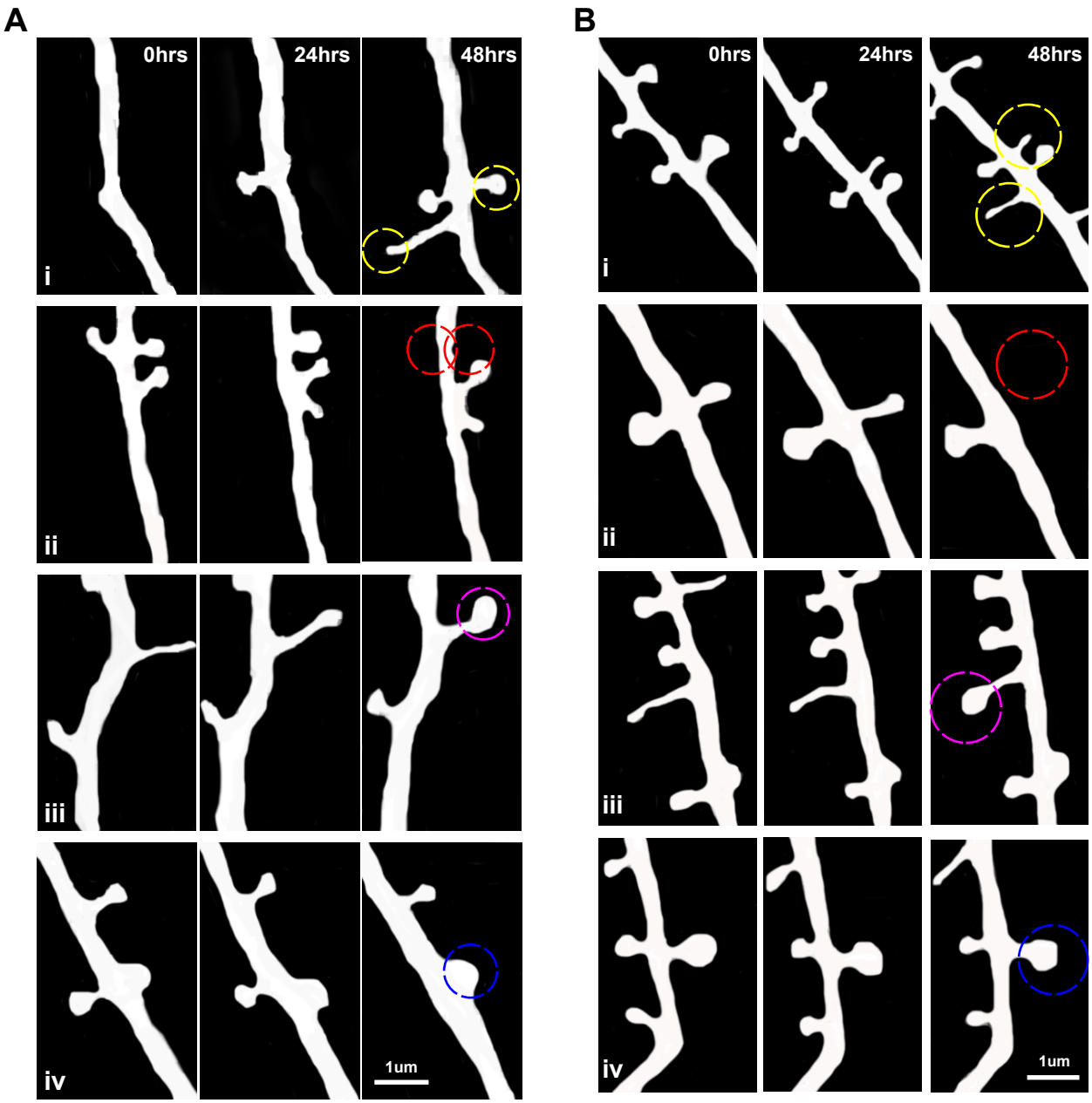
At the time point of P60, the male motor cortex displays a significantly ($p < 0.001$, Unpaired t-test) increased proportion of dynamic spines as compared to stable (37.84% ± 0.09 M SSC stable, 62.16% ± 0.09 M MC dynamic) (Figure 3.3.3 A). When split into the gain and loss of mature and immature spine types, the gain of mature spines was significantly ($p < 0.0001$, one-way ANOVA, Tukey's multiple comparison's test) greater than both the gain and the loss of immature spines (31.42% ± 0.05 M MC mature gain, 20.42% ± 0.03 M MC immature gain, 11.20% ± 0.04 M MC immature loss). Furthermore, the loss of mature spines also occurred at a significantly greater rate than the gain or loss of immature spine types (23.20% ± 0.06 M MC mature loss), indicative of specific mature spine turnover within the highly dynamic male motor cortex at P60 (Figure 3.3.3 B). Comparatively, in the male somatosensory cortex the proportion of stable spine types is significantly ($p < 0.001$, Unpaired t-test) greater than dynamic populations at this time point (69.75% ± 0.12 M SSC stable, 32.25% ± 0.10 M SSC dynamic) (Figure 3.3.3 C), with no significant differences in mature and immature spine turnover (Figure 3.3.3 D).

3.3.3 Cycling oestrogen stabilises dendritic spines within the motor cortex of female *Thy-YFP* mice at P60

To investigate the proportions of stable and dynamic spines within the female *Thy1-YFP* cortex, mice were split into groups experiencing either high or baseline oestrus levels, as determined by vaginal

Figure 3.3.2

(A, B) Representative images of dendritic spine dynamics in the 60 day (A) and 90 day (B) *Thy1-YFP* cortex, with turnover events defined as a gain (i); a loss (ii); and morphological maturation or dematuration (iii) over the 3 consecutive imaging sessions. Stable spine types (A iv) were those unchanged from each imaging round. Scale bar = 1μm.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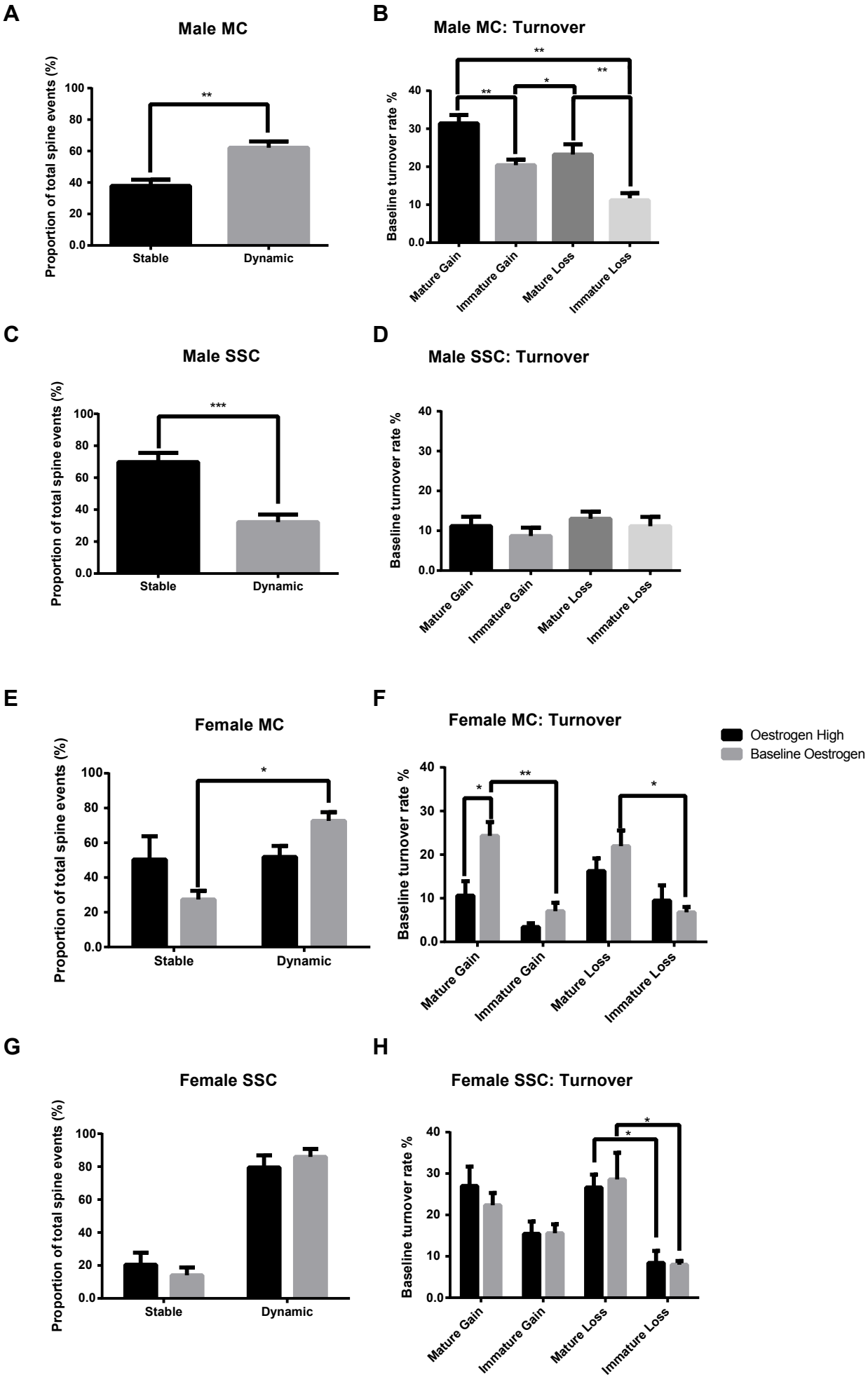
1um

Figure 3.3.3

Comparisons of stable and dynamic populations of dendritic spines within the *Thy1-YFP* male and female motor and somatosensory cortices at P60. Stable spines were quantified as those unchanged over the three imaging rounds, while dynamic spines underwent a gain, loss or morphological change event from the first imaging round to the last. (A, C) The graphs show the proportion of stable and dynamic dendritic spines within the male somatosensory and motor cortices. (B, D) The graphs show the turnover rate of mature and immature spines within the male (n = 5) motor and somatosensory cortices. (E, G) The graphs show the proportion of stable and dynamic dendritic spines within the female motor and somatosensory cortices during high (n = 4) and baseline (n = 4) levels of oestrogen. (F, H) The graphs show the turnover rate of mature and immature spines within the female motor and somatosensory cortices during high and baseline levels of oestrogen. MC = motor cortex, SSC = somatosensory cortex. Bars show mean ± SEM. *p = <0.05.


A
Male MC
Proportion of total spine events (%)
100
80
60
40
20
0.0
**
Stable
Dynamic
B
Male MC: Turnover
Baseline turnover rate %
40
30
20
10
0.0
**
*
**
**
Mature Gain
Immature Gain
Mature Loss
Immature Loss
C
Male SSC
Proportion of total spine events (%)
***
Stable
Dynamic
D
Male SSC: Turnover
Baseline turnover rate %
Mature Gain
Immature Gain
Mature Loss
Immature Loss
E
Female MC
Proportion of total spine events (%)
*
Stable
Dynamic
F
Female MC: Turnover
Baseline turnover rate %
*
**
*
Mature Gain
Immature Gain
Mature Loss
Immature Loss
Oestrogen High
Baseline Oestrogen
G
Female SSC
Proportion of total spine events (%)
Stable
Dynamic
H
Female SSC: Turnover
Baseline turnover rate %
*
*
Mature Gain
Immature Gain
Mature Loss
Immature Loss

lavage (Figure 3.2.1 Ai-v). At each imaging round, a snapshot was taken of morphology and the presence or absence of dendritic spines during either high oestrogen levels or during baseline levels.

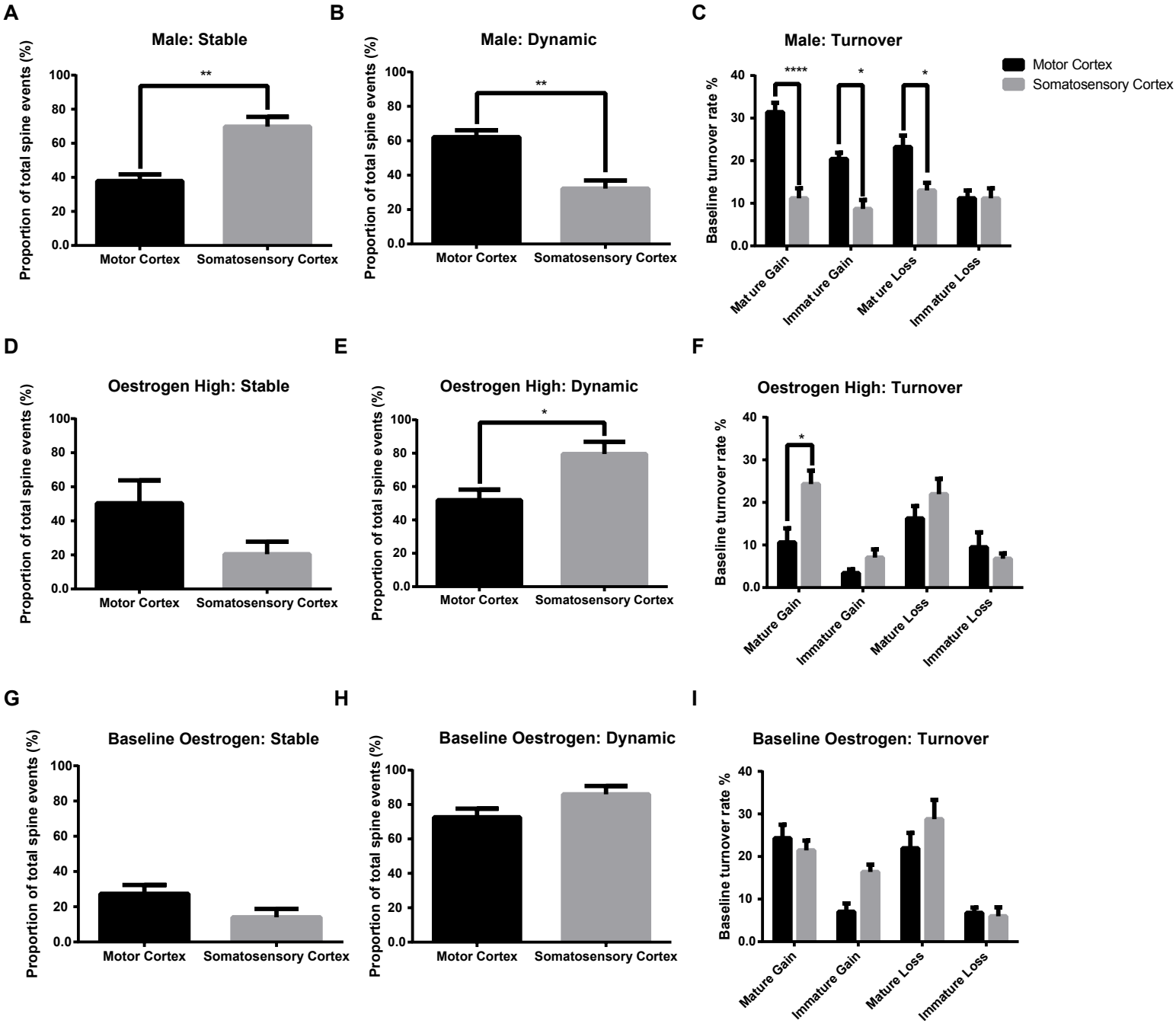
When experiencing high levels of the sex hormone oestrogen, mice displayed no significant differences between stable and dynamic dendritic spine proportions within the motor cortex; however, during baseline levels of oestrogen, there was a significant ($p < 0.02$, two-way ANOVA, Tukey's multiple comparisons test) increase in the dynamic proportion (27.40% ± 0.10 F baseline MC, 72.6% ± 0.10 F high MC) (Figure 3.3.3 E). Within the dynamic population, baseline oestrus induced significantly ($p < 0.05$, two-way ANOVA, Tukey's multiple comparisons test) increased mature spine gain as compared to immature gain during baseline oestrus (24.30% ± 0.06 F baseline MC mature gain, 7.00% ± 0.03 F baseline MC immature gain), and mature gain during high oestrus (10.60% ± 0.06 F high MC mature gain). The loss of mature spine types during baseline oestrus was also significantly greater than that of immature spine loss (22.00% ± 0.07 F baseline MC mature loss, 6.80% ± 0.02 F baseline MC immature loss) (Figure 3.3.3 F), and suggests that turnover during baseline oestrogen is similar to the increased turnover of mature spines within the male *Thy1-YFP* motor cortex at P60. There were no significant differences between stable and dynamic proportions of dendritic spines within the somatosensory cortex regardless of oestrogen levels (Figure 3.3.3 G). However, within the dynamic spine proportion both high and baseline oestrus states undergo significantly ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) greater loss of mature spine types compared to the loss of immature spine types (26.70% ± 0.06 F high SSC mature loss, 8.40% ± 0.06 F high SSC immature loss, 28.60% ± 0.11 F baseline SSC mature loss, 8.00% ± 0.01 F baseline SSC immature loss) (Figure 3.3.3 H). These findings highlight the role cycling of oestrogen plays in regulating the ratio of stable and dynamic dendritic spine proportions, with greater stability evident during high oestrogen.

3.3.4 Dendritic spines of the male *Thy1-YFP* motor cortex are more dynamic than the somatosensory cortex at P60

To further elucidate region-specific dendritic spine dynamics regulating neuronal networks, the motor and somatosensory cortices were compared within male and female *Thy1-YFP* mice at P60. Within the male *Thy1-YFP* cortex, the motor cortices display significantly ($p < 0.001$, Unpaired t-test) reduced stable populations as compared to the somatosensory cortex (37.84% ± 0.09 M MC stable, 69.75% ± 0.13 M SSC stable) (Figure 3.3.4 A) and a significantly ($p < 0.008$, Unpaired t-test) greater dynamic proportion (62.16% ± 0.32 M MC dynamic, 32.22% ± 0.10 M SSC dynamic) (Figure 3.3.4 B). Within the dynamic dendritic spine population of males, the gain and loss rates of mature spine types was

Figure 3.3.4

Comparisons of stable and dynamic populations of dendritic spines between the motor and somatosensory cortices of male and female *Thy1-YFP* mice at P60. (A, D, G) The graphs show the proportions of stable dendritic spines within the male (n = 5), high oestrogen female (n = 4) and baseline oestrogen (n = 4) motor and somatosensory cortices. (B, E, H) The graphs show the proportions of stable dendritic spines within the male, high oestrogen female and baseline oestrogen motor and somatosensory cortices. (C, F, I) The graphs show the gain and loss rates of mature and immature spines within the male high oestrogen female and baseline oestrogen motor and somatosensory cortices. MC = motor cortex, SSC = somatosensory cortex. Bars show mean ± SEM. *p = <0.05.


A
Male: Stable
Proportion of total spine events (%)
**
Motor Cortex
Somatosensory Cortex
B
Male: Dynamic
Proportion of total spine events (%)
**
Motor Cortex
Somatosensory Cortex
C
Male: Turnover
Baseline turnover rate %
****
*
*
Motor Cortex
Somatosensory Cortex
Mature Gain
Immature Gain
Mature Loss
Immature Loss
D
Oestrogen High: Stable
Proportion of total spine events (%)
Motor Cortex
Somatosensory Cortex
E
Oestrogen High: Dynamic
Proportion of total spine events (%)
*
Motor Cortex
Somatosensory Cortex
F
Oestrogen High: Turnover
Baseline turnover rate %
*
Mature Gain
Immature Gain
Mature Loss
Immature Loss
G
Baseline Oestrogen: Stable
Proportion of total spine events (%)
Motor Cortex
Somatosensory Cortex
H
Baseline Oestrogen: Dynamic
Proportion of total spine events (%)
Motor Cortex
Somatosensory Cortex
I
Baseline Oestrogen: Turnover
Baseline turnover rate %
Mature Gain
Immature Gain
Mature Loss
Immature Loss

significantly ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) greater in the motor cortex compared to the somatosensory cortex (31.40% ± 0.05 M MC mature gain, 11.20% ± 0.05 M SSC mature gain, 23.2% ± 0.06 M MC mature loss, 13.00% ± 0.04 M SSC mature loss). The gain rates of immature spines within the dynamic dendritic spine population of males was also higher than that of the somatosensory cortex (20.40% ± 0.03 M MC immature gain, 8.70% M SSC immature gain) (Figure 3.3.4 C).

No significant differences in stable dendritic spine populations were observed in the female *Thy1-YFP* motor and somatosensory cortices (Figure 3.3.4 D, G). However, during high oestrus states the somatosensory cortex has a significantly ($p < 0.02$, Unpaired t-test, Tukey's multiple comparisons test) greater dynamic population of dendritic spines as compared to the motor cortex (51.91% ± 0.80 F high MC dynamic, 79.56% ± 0.14 F high SSC dynamic) (Figure 3.3.4 E). This may be attributed to the significantly ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) increased gain rate of mature spines in the somatosensory cortex compared to the motor cortex (10.60% ± 0.06 F high MC mature gain, 24.30 ± 0.06 F high SSC mature gain) (Figure 3.3.4 D). No significant differences were identified between the motor and somatosensory cortices in the dynamic population and turnover of dendritic spines during baseline oestrogen (Figure 3.3.4 H, I). In accordance with the results above, these inter-regional comparisons further support the finding of greater mature spine turnover within the male motor cortex and suggest a role for oestrogen levels in the maturation of dendritic spines.

3.3.5 Dendritic spines of the female *Thy1-YFP* somatosensory cortex are more dynamic than the male *Thy1-YFP* motor cortex at P60

It is well established that gonadal hormones exert a critical influence on the structure and function of the brain during development and adulthood, and the sex hormone oestrogen has specifically been identified as a robust modulator of dendritic spine density (Alexander et al 2018, Brinton 2008, Gould et al 1990, Srivastava et al 2013, Woolley et al 1990). The findings thus far from the current chapter indicate sex-specific changes at the dendritic spine; to further investigate the role of sex, stable and dynamic dendritic spines proportions were compared between males and females experiencing high and baseline oestrus levels.

Within the motor cortex, there were no significant differences in the proportion of stable spines between groups (Figure 3.3.5 A). However, baseline levels of oestrogen in females resulted in a significantly ($p < 0.05$, two-way ANOVA, Tukey's multiple comparisons test) increased dynamic population of dendritic spines compared to females during high oestrogen, with no significance

compared to males (51.91% ± 0.12 F high MC dynamic, 72.62% ± 0.10 F baseline MC dynamic) (Figure 3.3.5 B). Within the dynamic proportions of dendritic spines, the gain rate of mature spines was significantly ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) reduced during high oestrogen when compared to females during baseline oestrogen and male *Thy1-YFP* mice (10.60% ± 0.06 F high MC mature gain, 24.30% ± 0.06 F baseline MC mature gain, 31.9% ± 0.05 M MC mature gain). During high oestrus the gain of immature spines was also significantly less than that of the male motor cortex (3.40% ± 0.02 F high MC immature gain, 20.30% ± 0.04 M MC immature gain) (Figure 3.3.5 C).

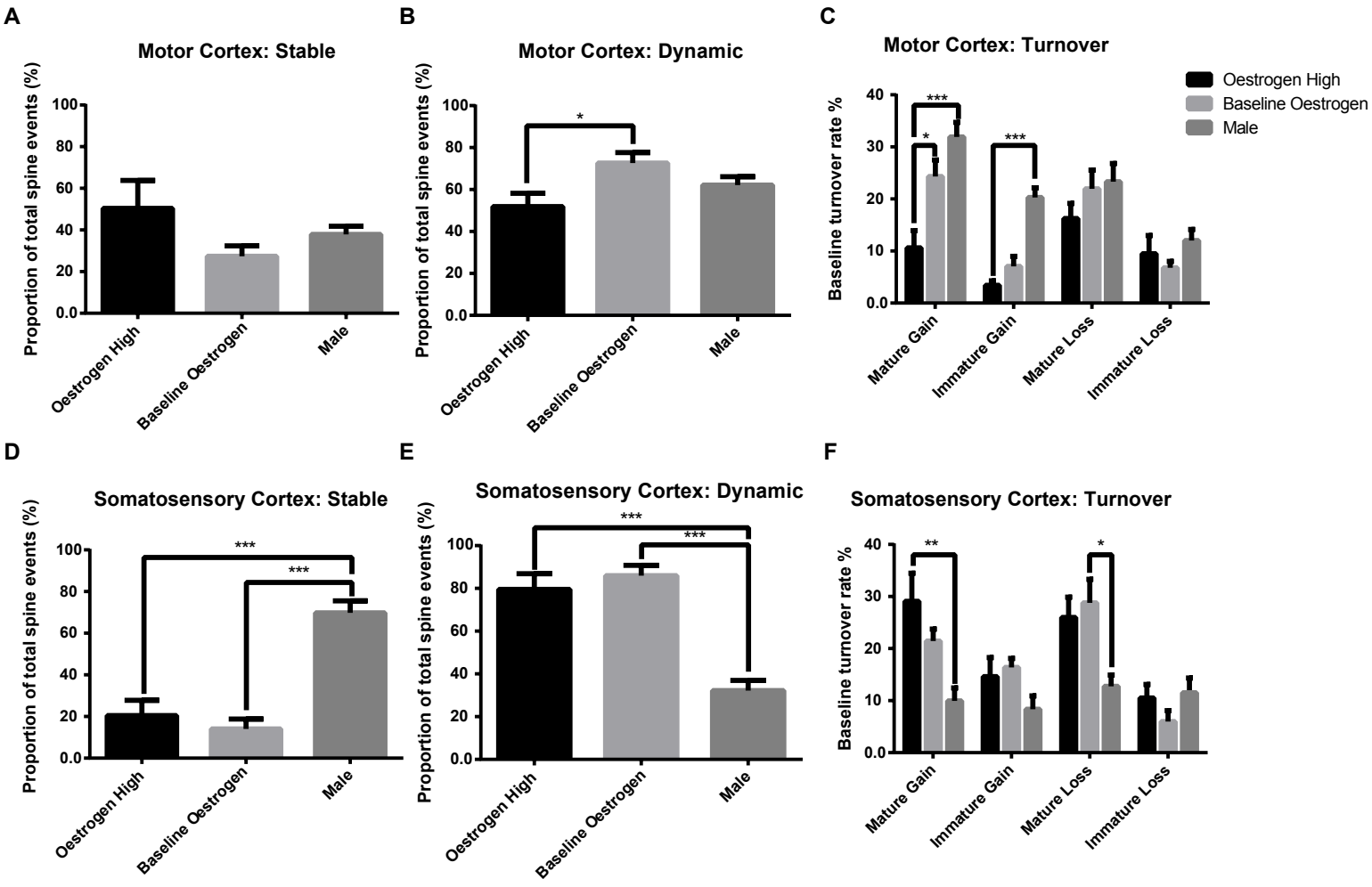
Within the somatosensory cortex male mice display a significantly ($p < 0.0001$, one-way ANOVA, Tukey's multiple comparisons test) higher proportion of stable spine types compared to females experiencing both high and baseline levels of oestrogen (20.44% ± 0.14 F high SSC stable, 14.01% ± 0.09 F baseline SSC stable, 69.75% ± 0.12 M SSC stable) (Figure 3.3.5 D). Accordingly, the proportion of dynamic spine populations in the male somatosensory cortex was significantly reduced compared to females (79.56% ± 0.14 F high SSC dynamic, 86.00% ± 0.09 F baseline SSC dynamic, 32.25% ± 0.10 M SSC dynamic) (Figure 3.3.5 E). Within dynamic proportions, high oestrogen levels are associated with significantly ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) higher mature spine gain as compared to the male somatosensory cortices (29.10% ± 0.11 F high SSC mature gain, 10.00% ± 0.05 M SSC mature gain), whilst baseline oestrogen levels are associated with significantly higher mature spine loss as compared to male somatosensory cortices (28.80% ± 0.09 F baseline SSC mature loss, 12.70% ± 0.05 M SSC mature loss) (Figure 3.3.5 F). These findings highlight the dynamics of the male motor cortex as compared to the stability of the male somatosensory cortex, and the increased dynamics of the female somatosensory cortex, further establishing sex-specific dendritic spine changes within cortical regions at P60.

3.3.6 Dendritic spines of the male *Thy1-YFP* motor and somatosensory cortices are highly dynamic at P90

To address one of the key research questions of this Chapter and to understand how development into adulthood may alter dendritic spines in the male and female *Thy1-YFP* motor and somatosensory cortices, 2PLSM was undertaken at P90. The same protocol was performed as described above (Figure 3.3.2). At P90, there were no significant differences between stable and dynamic populations of dendritic spines within the male motor cortex (Figure 3.3.6 A). However, within the dynamic dendritic spine proportion there was a significant ($p < 0.0006$, one-way ANOVA, Tukey's multiple comparisons test) increase in mature spine gain rates compared to the overall turnover of immature spines (23.29%

Figure 3.3.5

Comparisons of stable and dynamic populations of dendritic spines between male and female *Thy1-YFP* motor and somatosensory cortices at P60. Stable spines were quantified as those unchanged over the three imaging rounds, while dynamic spines underwent a gain, loss or morphological change event from the first imaging round to the last. (A, D) The graphs show the proportion of stable dendritic spines within the motor and somatosensory cortices of male ($n = 5$) mice and female mice experiencing high ($n = 4$) and baseline ($n = 4$) levels of oestrogen. (B, E) The graphs show the proportion of dynamic dendritic spines within the motor and somatosensory cortices of male and female mice experiencing high and baseline levels of oestrogen. (C, F) The graphs show the turnover of mature and immature spines within the motor and somatosensory cortices male and female mice experiencing high and baseline levels of oestrogen. MC = motor cortex, SSC = somatosensory cortex. Bars show mean ± SEM. $*p = <0.05$.


A
Motor Cortex: Stable
Proportion of total spine events (%)
100
80
60
40
20
0.0
Oestrogen High
Baseline Oestrogen
Male
B
Motor Cortex: Dynamic
Proportion of total spine events (%)
*
Oestrogen High
Baseline Oestrogen
Male
C
Motor Cortex: Turnover
Baseline turnover rate %
40
30
20
10
0.0
***
*
***
Mature Gain
Immature Gain
Mature Loss
Immature Loss
Oestrogen High
Baseline Oestrogen
Male
D
Somatosensory Cortex: Stable
Proportion of total spine events (%)
***
***
Oestrogen High
Baseline Oestrogen
Male
E
Somatosensory Cortex: Dynamic
Proportion of total spine events (%)
***
***
Oestrogen High
Baseline Oestrogen
Male
F
Somatosensory Cortex: Turnover
Baseline turnover rate %
**
*
Mature Gain
Immature Gain
Mature Loss
Immature Loss

± 0.02 M MC mature gain, 5.52% ± 0.03 M MC immature gain, 8.45% ± 0.05 M MC immature loss) (Figure 3.3.6 B). The loss of mature spines was also significantly increased, occurring at a greater rate than the overall turnover of immature spines (27.53% ± 0.02 M MC mature loss) (Figure 3.3.6 B). Within the male somatosensory cortices at P90, the dynamic dendritic spine proportion was significantly ($p < 0.006$, Unpaired t-test) greater than that of the stable dendritic spine proportion (37.35% ± 0.12 M SSC stable, 62.65% ± 0.12 M SSC dynamic) (Figure 3.3.6 C). Within the dynamic dendritic spine proportion, there was a significant ($p < 0.003$, one-way ANOVA, Tukey's multiple comparisons test) increase in mature spine gain compared to the overall turnover of immature spines (26.88% ± 0.08 M SSC mature gain, 8.60% ± 0.1 M SSC immature gain, 7.56% ± 0.03 M SSC immature loss) (Figure 3.3.6 D). The loss of mature spines was also significantly increased, occurring at a greater rate than the overall turnover of immature spines (22.05% ± 0.04 M SSC mature loss) (Figure 3.3.6 D). These findings highlight the maintained high dynamics of the motor cortex, as well as the potentially increased dynamics of the somatosensory cortex.

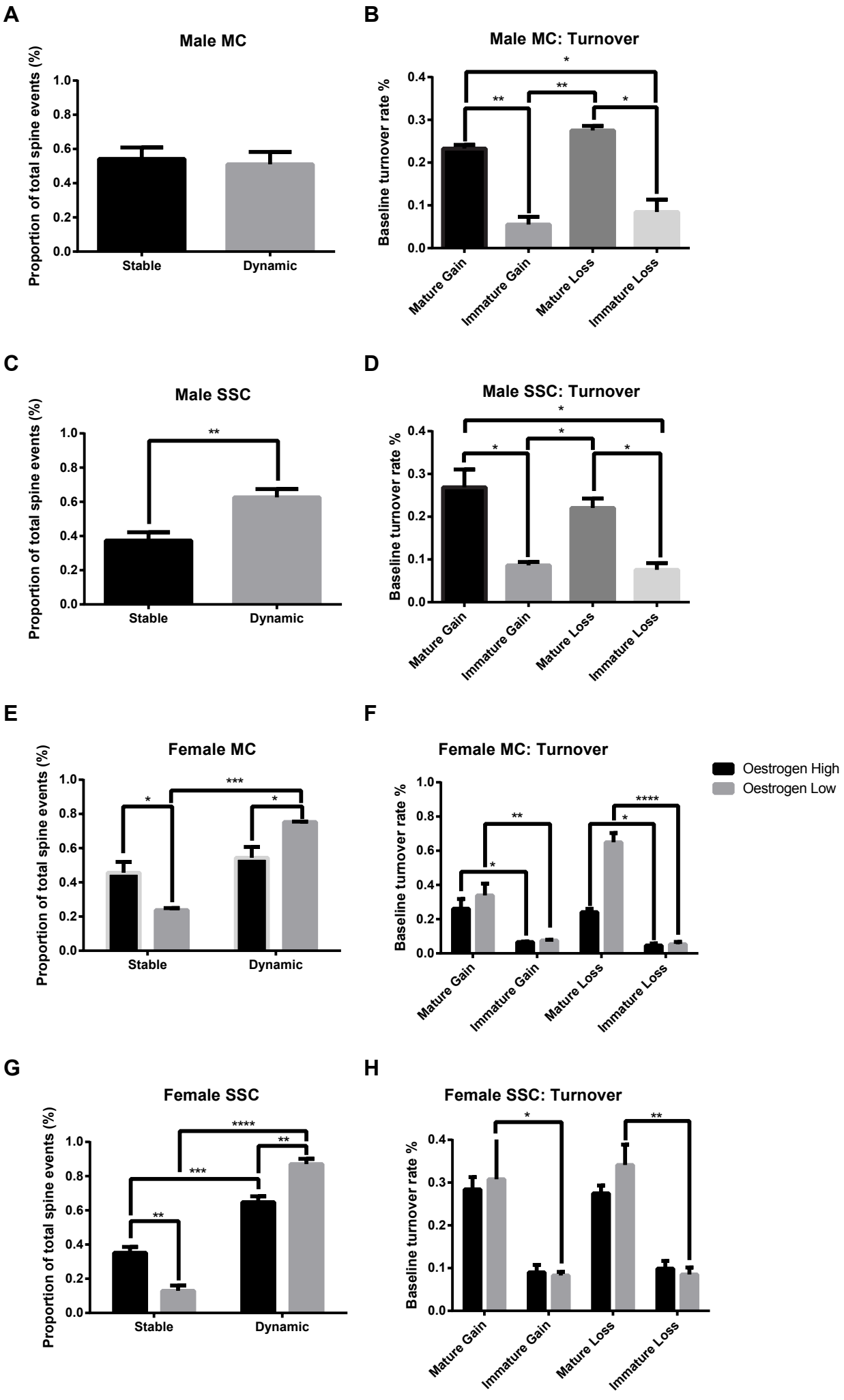
3.3.7 Dendritic spines of the female *Thy1-YFP* somatosensory cortex are highly dynamic and mature spine turnover influenced by cycling oestrogen at P90

At P90 in the female *Thy1-YFP* motor cortex, high oestrus mice display a significantly ($p < 0.0015$, two-way ANOVA, Tukey's multiple comparisons test) greater stable dendritic spine population than female mice during baseline oestrogen (45.60% ± 0.11 F high MC stable, 23.80% ± 0.02 F baseline MC stable). Females experiencing baseline oestrogen levels have a greater dynamic dendritic spine population than stable dendritic spine population (54.30% ± 0.11 F high MC dynamic, 75.3% ± 0.01 F baseline MC dynamic), and a higher dynamic dendritic spine population than females experiencing high oestrogen levels (Figure 3.3.6 E). Within the dynamic proportions, females experiencing both high and baseline levels of oestrogen display a significantly ($p < 0.0002$, two-way ANOVA, Tukey's multiple comparisons test) greater gain rate of mature spines as compared to the gain rate of immature spines (26.20% ± 0.09 F high MC mature gain, 6.70% ± 0.05 F high MC immature gain, 33.70% ± 0.12 F baseline MC mature gain, 7.50% ± 0.08 F baseline MC immature gain), as well as a greater loss rate of mature spines compared to the loss rate of immature spines (24.10% ± 0.03 F high MC mature loss, 4.60% ± 0.02 F high MC immature loss, 65.00% ± 0.09 F baseline MC mature loss, 5.30% ± 0.02 F baseline MC immature loss) (Figure 3.3.6 F).

Within the P90 female *Thy1-YFP* somatosensory cortex, the proportion of dynamic spines is significantly ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) greater than that of stable spines regardless of oestrogen levels (35.20% ± 0.06 F high SSC stable, 64.8% ± 0.06 F high

Figure 3.3.6

Comparisons of stable and dynamic populations of dendritic spines within the *Thy1-YFP* male and female motor and somatosensory cortices at P90. (A, C) The graphs show the proportion of stable and dynamic dendritic spines within the male somatosensory and motor cortices. (B, D) The graphs show the turnover rate of mature and immature spines within the male (n = 5) motor and somatosensory cortices. (E, G) The graphs show the proportion of stable and dynamic dendritic spines within the female motor and somatosensory cortices during high (n = 3) and baseline (n = 3) levels of oestrogen. (F, H) The graphs show the turnover rate of mature and immature spines within the female motor and somatosensory cortices during high and baseline levels of oestrogen. MC = motor cortex, SSC = somatosensory cortex. Bars show mean ± SEM. *p = <0.05.


A
Male MC
Proportion of total spine events (%)
1.0
0.8
0.6
0.4
0.2
0.0
Stable
Dynamic
B
Male MC: Turnover
Baseline turnover rate %
0.4
0.3
0.2
0.1
0.0
*
**
**
*
Mature Gain
Immature Gain
Mature Loss
Immature Loss
C
Male SSC
Proportion of total spine events (%)
**
Stable
Dynamic
D
Male SSC: Turnover
Baseline turnover rate %
*
*
*
*
Mature Gain
Immature Gain
Mature Loss
Immature Loss
E
Female MC
Proportion of total spine events (%)
*
***
*
Stable
Dynamic
F
Female MC: Turnover
Baseline turnover rate %
**
*
****
*
Mature Gain
Immature Gain
Mature Loss
Immature Loss
Oestrogen High
Oestrogen Low
G
Female SSC
Proportion of total spine events (%)
****
**
***
**
Stable
Dynamic
H
Female SSC: Turnover
Baseline turnover rate %
*
**
Mature Gain
Immature Gain
Mature Loss
Immature Loss

SSC dynamic, 13.00% ± 0.05 F baseline SSC stable, 87.00% ± 0.05 F baseline SSC dynamic) (Figure 3.3.6 G). Between high and baseline oestrogen levels, there was a significantly higher proportion of stable spines at high oestrus than those during baseline oestrus; conversely, during baseline oestrus the dynamic dendritic spine populations were greater than those at high oestrus (Figure 3.3.6 G). Within the dynamic proportions during baseline oestrogen, there was significantly ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) greater overall turnover of mature spines compared to immature spines (30.80% ± 0.16 F baseline SSC mature gain, 8.30% ± 0.01 F baseline SSC immature gain, 34.10% ± 0.08 F baseline SSC mature loss, 8.50% ± 0.03 F baseline SSC immature loss) (Figure 3.3.6 H). When considered as a whole, the data suggests that at P90, cycling oestrogen is associated with the alternating proportion of stable and dynamic spines within the motor and somatosensory cortices.

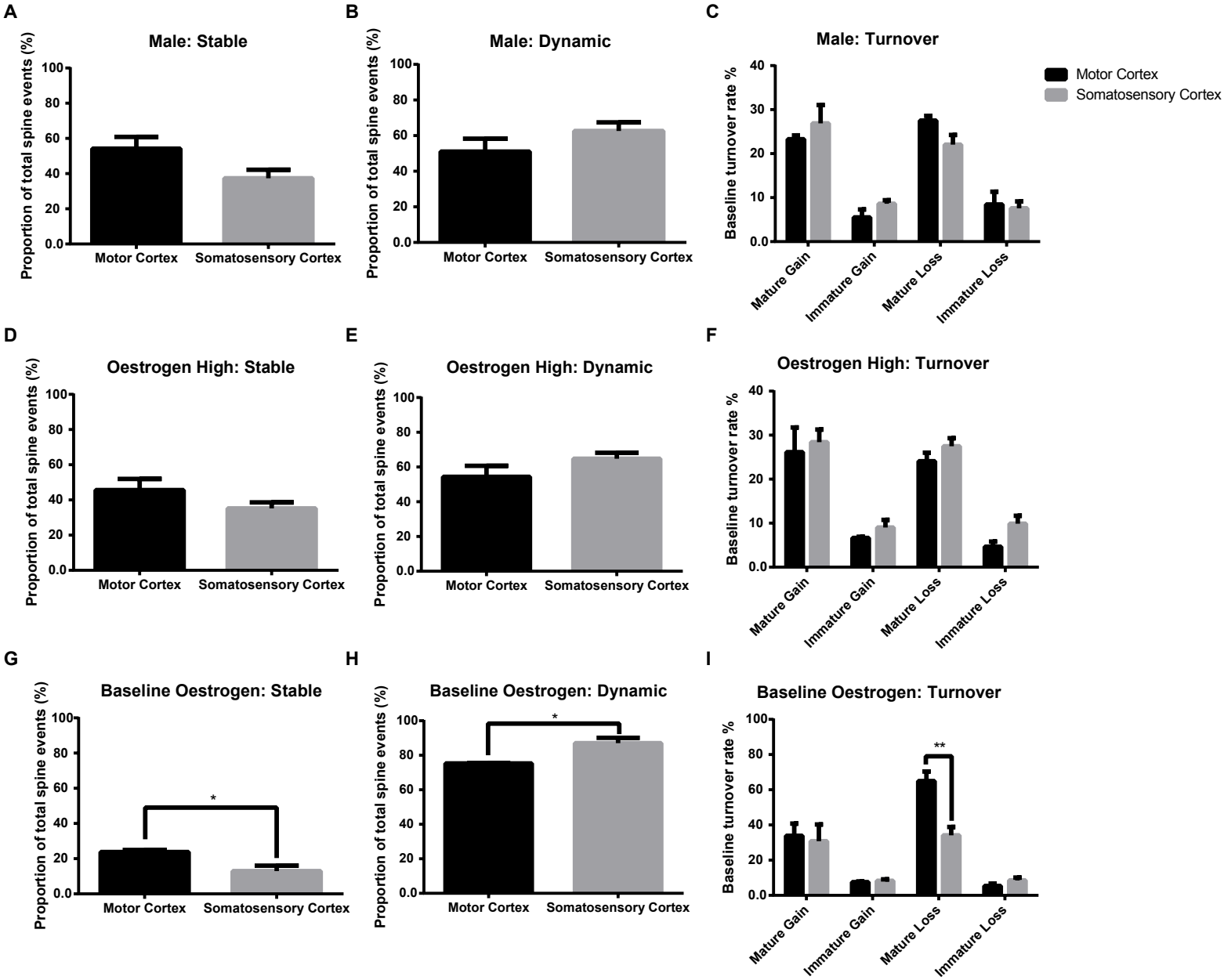
3.3.8 Baseline levels of oestrogen are associated with regional dendritic spine changes in the female *Thy1-YFP* cortex at P90

To elucidate region-specific activity by adulthood, dendritic spine dynamics of the motor and somatosensory cortices were compared within male and female *Thy1-YFP* mice at P90. No significant differences were identified in the stable and dynamic proportions of dendritic spines within the male cortex, nor of that in the female cortex during high oestrogen (Figure 3.3.7 A, B, C, D, E, F). However, during baseline oestrus dendritic spines in the motor cortex were significantly ($p < 0.05$, Unpaired t-test) more stable than those within the somatosensory cortex (23.79% ± 0.02 F baseline MC stable, 12.96% ± 0.05 F baseline SSC stable) (Figure 3.3.7 G). Accordingly, the somatosensory cortex was significantly ($p < 0.01$, Unpaired t-test) more dynamic than the motor cortex during baseline oestrogen (75.31% ± 0.00 F baseline MC dynamic, 87.04 ± 0.05 F baseline SSC dynamic) (Figure 3.3.7 H). Intriguingly, within the dynamic populations of the female *Thy1-YFP* cortex during baseline oestrus, the motor cortex displays a significantly ($p < 0.05$, two-way ANOVA, Tukey's multiple comparisons test) increased rate of mature spine loss (65.00% ± 0.09 F baseline MC mature loss, 34.10% ± 0.08 F baseline SSC mature loss) (Figure 3.3.7 I). This finding has also been observed at P60 as detailed above, and may indicate oestrogen-regulated activity at mature dendritic spines.

To address sex-specific dendritic spine changes in the adult cortex, stable and dynamic populations were compared between females experiencing high and baseline oestrus states, and male YFPH mice. Within the motor cortex, no significant differences in stable dendritic spine populations were identified between high oestrus females and males; however, female mice experiencing baseline oestrogen displayed a significantly ($p < 0.05$, one-way ANOVA, Tukey's multiple comparisons test) reduced

Figure 3.3.7

Comparisons of stable and dynamic populations of dendritic spines between the motor and somatosensory cortices of male and female *Thy1-YFP* mice at P90. Stable spines were quantified as those unchanged over the three imaging rounds, while dynamic spines underwent a gain, loss or morphological change event from the first imaging round to the last. (A, D, G) The graphs show the proportions of stable dendritic spines within the male (n = 5), high oestrogen female (n = 3) and baseline oestrogen (n = 3) motor and somatosensory cortices. (B, E, H) The graphs show the proportions of stable dendritic spines within the male, high oestrogen female and baseline oestrogen motor and somatosensory cortices. (C, F, I) The graphs show the gain and loss rates of mature and immature spines within the male high oestrogen female and baseline oestrogen motor and somatosensory cortices. MC = motor cortex, SSC = somatosensory cortex. Bars show mean ± SEM. *p = <0.05.


A
Male: Stable
Proportion of total spine events (%)
100
80
60
40
20
0.0
Motor Cortex
Somatosensory Cortex
B
Male: Dynamic
Proportion of total spine events (%)
100
80
60
40
20
0.0
Motor Cortex
Somatosensory Cortex
C
Male: Turnover
Baseline turnover rate %
40
30
20
10
0.0
Mature Gain
Immature Gain
Mature Loss
Immature Loss
Motor Cortex
Somatosensory Cortex
D
Oestrogen High: Stable
Proportion of total spine events (%)
100
80
60
40
20
0.0
Motor Cortex
Somatosensory Cortex
E
Oestrogen High: Dynamic
Proportion of total spine events (%)
100
80
60
40
20
0.0
Motor Cortex
Somatosensory Cortex
F
Oestrogen High: Turnover
Baseline turnover rate %
40
30
20
10
0.0
Mature Gain
Immature Gain
Mature Loss
Immature Loss
G
Baseline Oestrogen: Stable
Proportion of total spine events (%)
100
80
60
40
20
0.0
*
Motor Cortex
Somatosensory Cortex
H
Baseline Oestrogen: Dynamic
Proportion of total spine events (%)
100
80
60
40
20
0.0
*
Motor Cortex
Somatosensory Cortex
I
Baseline Oestrogen: Turnover
Baseline turnover rate %
100
80
60
40
20
0.0
**
Mature Gain
Immature Gain
Mature Loss
Immature Loss

proportion of stable spines when compared to male *Thy1-YFP* mice at P90 (23.79% ± 0.02 F baseline MC stable, 54.24% ± 0.13 M MC stable) (Figure 3.3.8 A). No significant differences in dynamic dendritic spine proportions were observed between the sexes (Figure 3.3.8 B). However, within dynamic populations the loss of mature spines during baseline oestrogen in females was significantly ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) greater than at high oestrus, or within males (24.1% ± 0.3 F high MC mature loss, 65.00% ± 0.09 F baseline MC mature loss, 27.5% ± 0.02 M MC mature loss) (Figure 3.3.8 C). The proportion of stable spines was also comparatively reduced during baseline oestrogen levels, being significantly ($p < 0.05$, two-way ANOVA, Tukey's multiple comparisons test) lower than that of the male somatosensory cortex (14.01% ± 0.09 F baseline SSC stable, 37.35 ± 0.12 M SSC stable) (Figure 3.3.8 D). A significantly higher dynamic population of dendritic spines was also observed during baseline oestrogen when compared to high oestrus levels and males (64.78% ± 0.05 F high SSC dynamic, 87.04% ± 0.05 F baseline SSC dynamic, 62.65% ± 0.12 M SSC dynamic) (Figure 3.3.8 E). No significant differences were identified in overall dendritic spine turnover within the dynamic spine proportions between the sexes (Figure 3.3.8 F).

3.3.9 Dendritic spines of the male *Thy1-YFP* somatosensory cortex are increasingly dynamic during maturation

The aforementioned findings highlight specific alterations to mature spines at adulthood in the *Thy1-YFP* mouse model, with sex associated with differential dynamic and stable dendritic spine populations at P90. To determine the influence of ageing, dendritic spine changes within the motor and somatosensory cortices were compared between male and female mice at P60 and at P90.

Within the male motor cortex, no differences in the proportion of stable and dynamic dendritic spines were identified between P60 and P90 (Figure 3.3.9 A). However, within dynamic spine populations there was a significant ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) reduction in the gain rate of immature spines from P60 to P90 (20.4% ± 0.03 P60 M MC immature gain, 5.5% ± 0.03 P90 M MC immature gain) (Figure 3.3.9 B). Within the somatosensory cortices, there was a reversal of dynamic and stable proportions between the two age groups, where by P90 there was a significant ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) reduction in stable populations (69.8% ± 0.13 P60 M SSC stable, 37.5% ± 0.12 P90 M SSC stable) and a significant increase in dynamics populations (32.2% ± 0.10 P60 M SSC dynamic, 62.6% ± 0.12 P90 M SSC dynamic) as compared to those at P60 (Figure 3.3.9 C). Within the dynamic dendritic spine proportion, there was a significant ($p < 0.001$, two-way ANOVA, Tukey's multiple comparisons test) increase in the gain of mature spines from P60 to P90 in the male somatosensory cortex (11.2% ± 0.05 P60 M

Figure 3.3.8

Comparisons of stable and dynamic populations of dendritic spines between male and female *Thy1-YFP* motor and somatosensory cortices at P90. (A, C) The graphs show the proportion of stable dendritic spines within the motor and somatosensory cortices of male (n = 5) mice and female mice experiencing high (n = 3) and baseline (n = 3) levels of oestrogen. (B, D) The graphs show the turnover of mature and immature spines within the motor and somatosensory cortices male mice and female mice experiencing high (n = 3) and baseline (n = 3) levels of oestrogen. MC = motor cortex, SSC = somatosensory cortex. Bars show mean ± SEM. *p = <0.05.

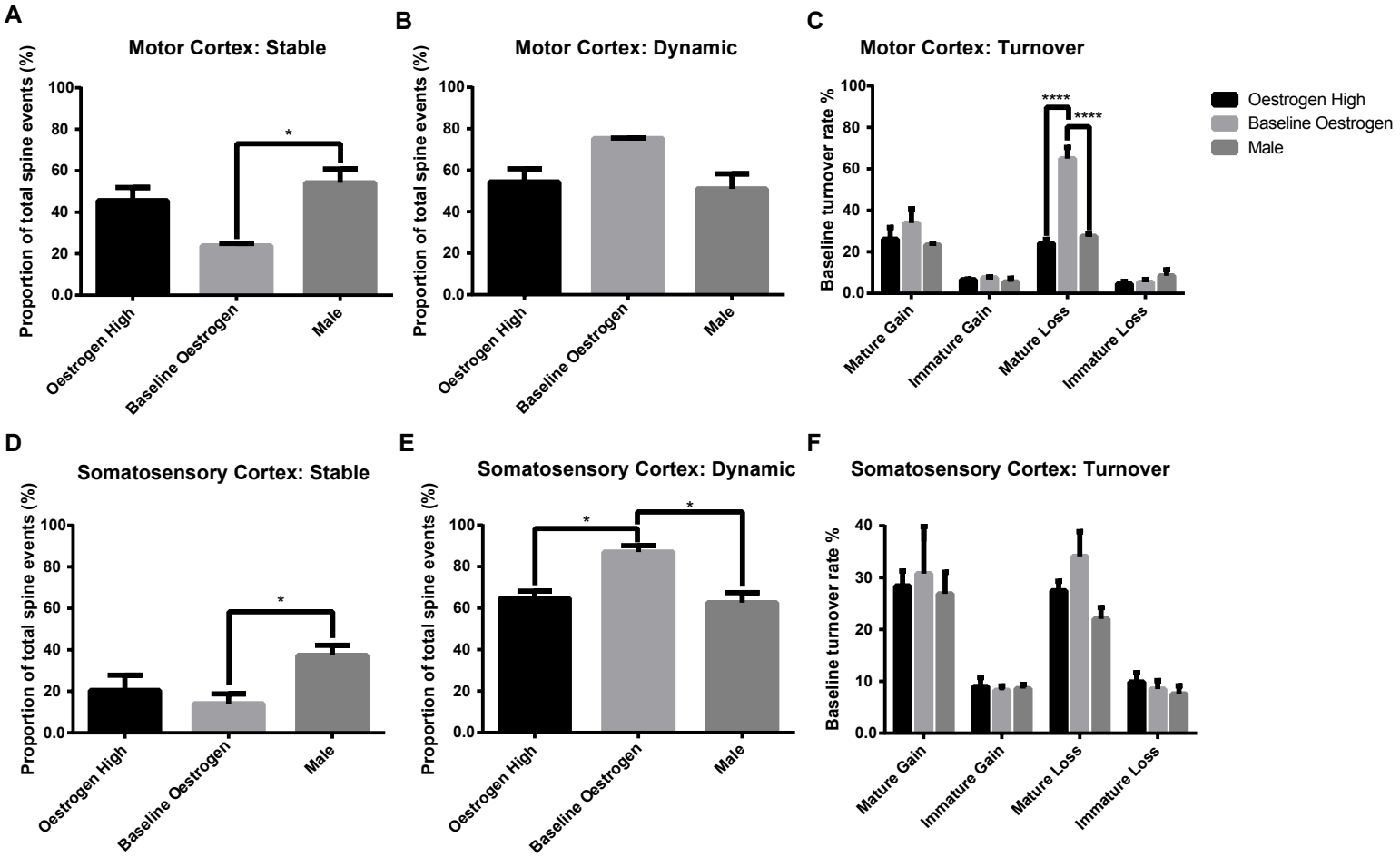
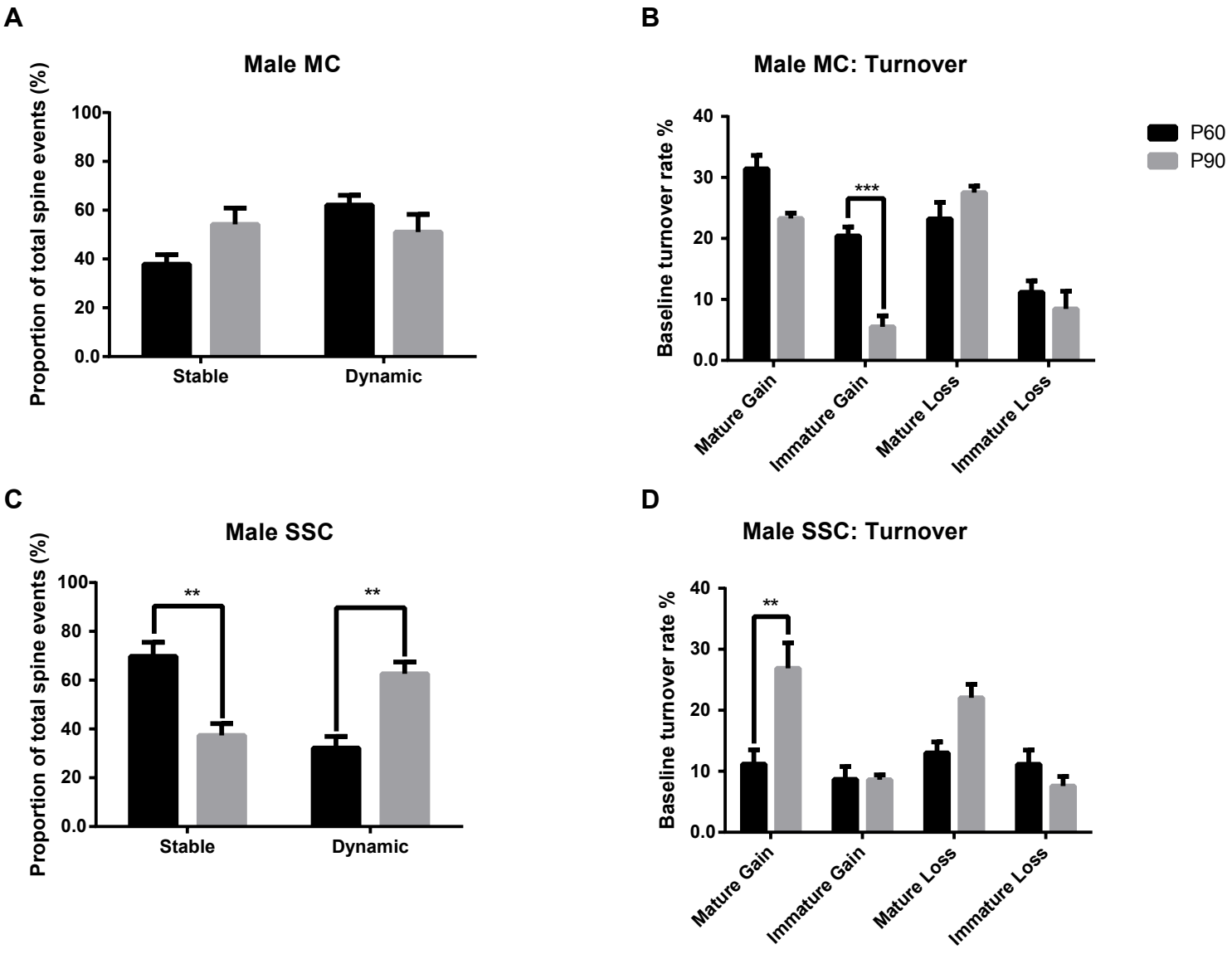

A
Motor Cortex: Stable
Proportion of total spine events (%)
100
80
60
40
20
0.0
*
Oestrogen High
Baseline Oestrogen
Male
B
Motor Cortex: Dynamic
Proportion of total spine events (%)
Oestrogen High
Baseline Oestrogen
Male
C
Motor Cortex: Turnover
Baseline turnover rate %
****
****
Mature Gain
Immature Gain
Mature Loss
Immature Loss
Oestrogen High
Baseline Oestrogen
Male
D
Somatosensory Cortex: Stable
Proportion of total spine events (%)
*
Oestrogen High
Baseline Oestrogen
Male
E
Somatosensory Cortex: Dynamic
Proportion of total spine events (%)
*
*
Oestrogen High
Baseline Oestrogen
Male
F
Somatosensory Cortex: Turnover
Baseline turnover rate %
40
30
20
10
0.0
Mature Gain
Immature Gain
Mature Loss
Immature Loss

Figure 3.3.9

Comparisons of stable and dynamic populations of dendritic spines between male *Thy1-YFP* motor and somatosensory cortices from P60 to P90. (A, C) The graphs show the proportion of stable dendritic spines within the motor and somatosensory cortices of male (n = 5) mice at P60 and at P90. (B, D) The graphs show the turnover of mature and immature spines within the motor and somatosensory cortices male (n = 5) mice at P60 and P90. MC = motor cortex, SSC = somatosensory cortex. Bars show mean ± SEM. *p = <0.05.


A
Male MC
Proportion of total spine events (%)
100
80
60
40
20
0.0
Stable
Dynamic
B
Male MC: Turnover
Baseline turnover rate %
40
30
20
10
0.0
***
P60
P90
Mature Gain
Immature Gain
Mature Loss
Immature Loss
C
Male SSC
Proportion of total spine events (%)
100
80
60
40
20
0.0
**
**
Stable
Dynamic
D
Male SSC: Turnover
Baseline turnover rate %
40
30
20
10
0.0
**
Mature Gain
Immature Gain
Mature Loss
Immature Loss

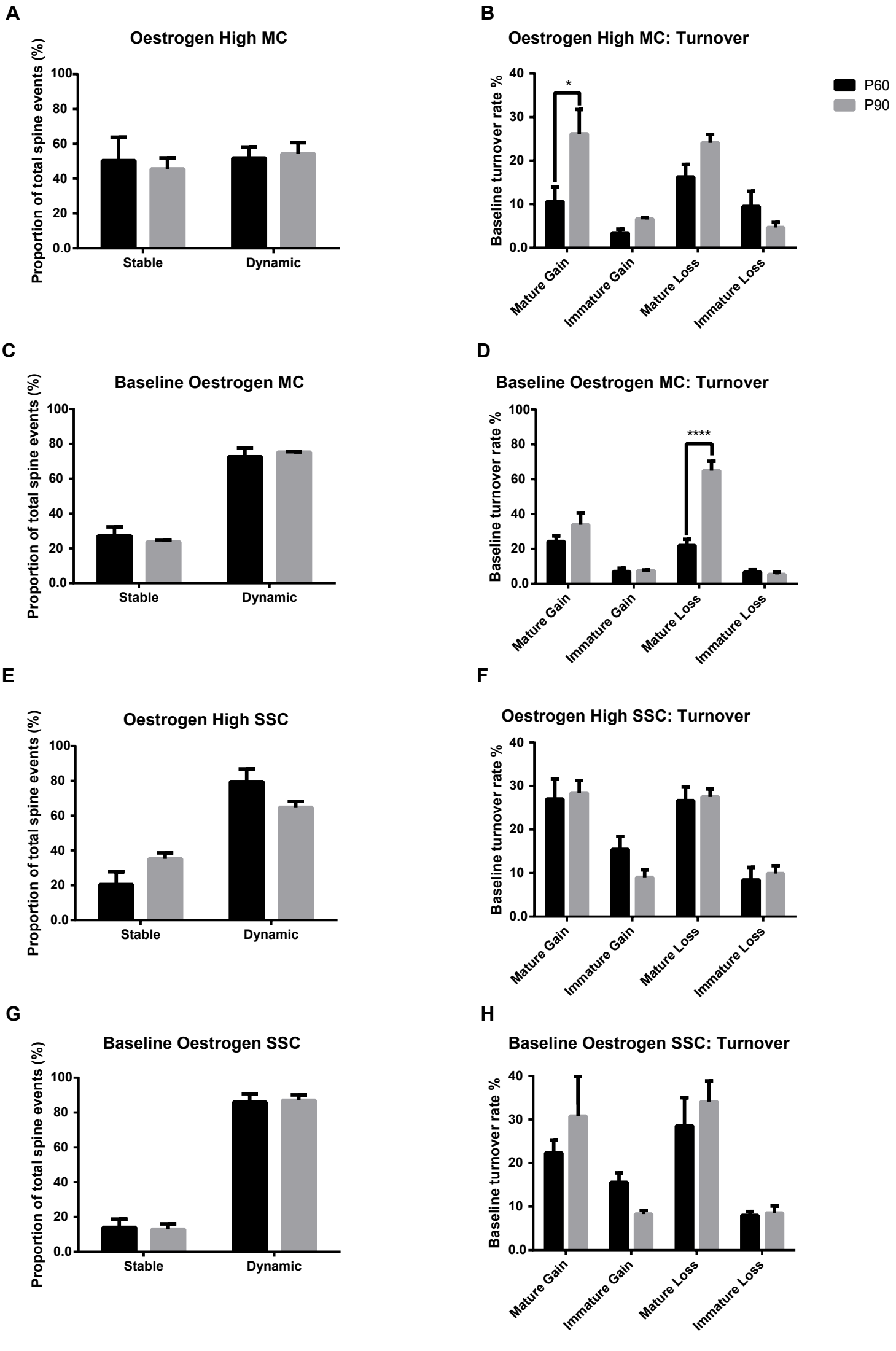
SSC mature gain, 26.9% ± 0.08 P90 M SSC mature gain) (Figure 3.3.9 D). These findings suggest that within the somatosensory cortex of male YFPH mice, the proportion of stabilisation and dynamic events of neuronal circuits is altered over time. Conversely, dynamics are maintained over time in the motor cortex, with evidence of maturation in the increased gain of mature spines within the somatosensory cortex and the reduced growth of immature spines in the motor cortex.

3.3.10 Cycling oestrogen influences increased mature spine turnover in the female *Thy1-YFP* motor cortex between P60 and P90

Between P60 and P90, there were no significant differences in the dynamic and stable dendritic spine proportions of the female motor and somatosensory cortices regardless of oestrogen levels (Figure 3.3.10 A, C, E, G). However, during high oestrogen levels the dynamic proportion of dendritic spines within the motor cortices displayed a significantly ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) increased gain rate of mature spines at P90 as compared to P60 (10.6% ± 0.06 P60 MC F high mature gain, 26.2% ± 0.09 P90 MC F high mature gain) (Figure 3.3.10 B). Conversely, during baseline levels of oestrogen there was a significant ($p < 0.0001$, two-way ANOVA, Tukey's multiple comparisons test) increase in mature spine loss within the motor cortex at P90 as compared to P60 (22.00% ± 0.07 P60 F baseline MC mature loss, 65.00% ± 0.09 P90 F baseline MC mature loss) (Figure 3.3.10 D). No significant differences were identified in turnover within the dynamic populations of the somatosensory cortex during high or baseline oestrogen levels (Figure 3.3.10 H). Thus the motor cortex displays age-dependent changes at mature dendritic spine types, while within the female somatosensory cortex dendritic spine populations are maintained from P60 to P90.

Figure 3.3.10

Comparisons of stable and dynamic populations of dendritic spines between female *Thy1-YFP* motor and somatosensory cortices during high and baseline oestrogen levels from P60 to P90. (A, C) The graphs show the proportion of stable dendritic spines within the motor cortices of female (n = 5) mice at P60 and at P90 during high and baseline oestrogen levels. (B, D) The graphs show the turnover of mature and immature spines within the motor cortices of female (n = 5) mice at P60 and P90 during high and baseline oestrogen levels. (E, G) The graphs show the proportion of stable dendritic spines within the somatosensory cortices of female (n = 5) mice at P60 and at P90 during high and baseline oestrogen levels. (F, H) The graphs show the turnover of mature and immature spines within the somatosensory cortices of female (n = 5) mice at P60 and P90 during high and baseline oestrogen levels. MC = motor cortex, SSC = somatosensory cortex. Bars show mean ± SEM. *p = <0.05.


A
Oestrogen High MC
Proportion of total spine events (%)
100
80
60
40
20
0.0
Stable
Dynamic
B
Oestrogen High MC: Turnover
Baseline turnover rate %
40
30
20
10
0.0
*
P60
P90
Mature Gain
Immature Gain
Mature Loss
Immature Loss
C
Baseline Oestrogen MC
Proportion of total spine events (%)
100
80
60
40
20
0.0
Stable
Dynamic
D
Baseline Oestrogen MC: Turnover
Baseline turnover rate %
100
80
60
40
20
0.0
****
Mature Gain
Immature Gain
Mature Loss
Immature Loss
E
Oestrogen High SSC
Proportion of total spine events (%)
100
80
60
40
20
0.0
Stable
Dynamic
F
Oestrogen High SSC: Turnover
Baseline turnover rate %
40
30
20
10
0.0
Mature Gain
Immature Gain
Mature Loss
Immature Loss
G
Baseline Oestrogen SSC
Proportion of total spine events (%)
100
80
60
40
20
0.0
Stable
Dynamic
H
Baseline Oestrogen SSC: Turnover
Baseline turnover rate %
40
30
20
10
0.0
Mature Gain
Immature Gain
Mature Loss
Immature Loss

3.4 DISCUSSION

The formation and modulation of the CNS relies upon precisely regulated synaptic circuits to function correctly. These cortical networks are assembled during development within discrete, yet interconnected regions through the formation and remodelling of dendritic spines (Katz & Shatz 1996). The current study aimed to determine whether cortical region, age and sex influence dendritic spine changes in the *Thy1-YFP* mouse model, and whether these may underlie differential susceptibility to disease. It was hypothesised that changes in dendritic spine populations and turnover have a unique pattern depending on these factors. Here, an increase in dendritic spine density is identified at P60 in both the motor and somatosensory cortices, indicative of a previously uncharacterised peak in neuroplastic capacity during adolescence. During this stage, male and female *Thy1-YFP* mice display a higher turnover of dendritic spines in the motor cortex, with peaks of high oestrogen associated with transient stability. The high dynamics of the male motor cortex are maintained into adulthood, as are the oestrogen-mediated changes in the female motor cortex. Within the somatosensory cortex, a switch between stability and dynamics is observed in males over time, whilst females sustain their relatively high proportions of dynamic dendritic spine populations (Figure 3.4.1).

3.4.1 Peaks of neuronal plasticity in the maturing cortex

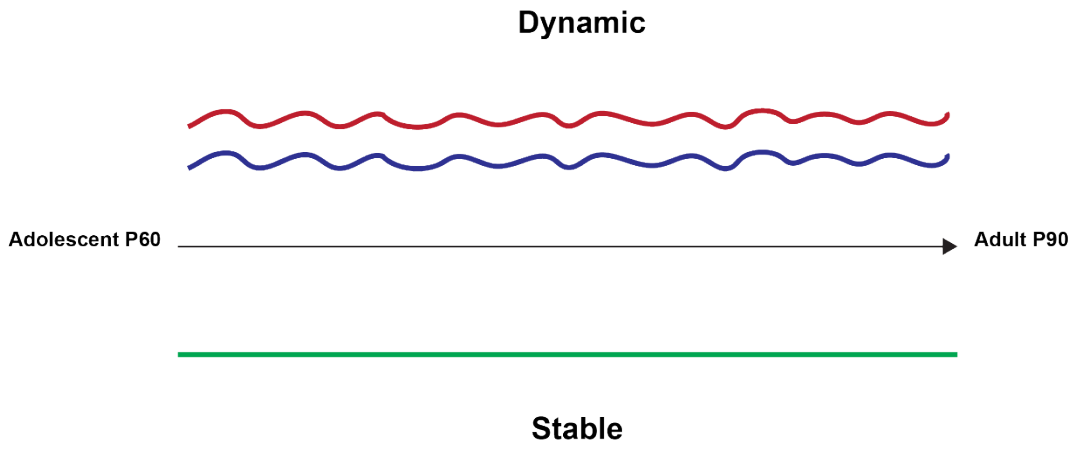
Although there is evidence for changes in overall dendritic spine density throughout cortical development, little is still known regarding the specific changes that occur at the highly motile postsynaptic structure in the mouse cortex over time, as influenced by sex (Bhatt et al 2009, Zuo et al 2005). Peaks in spine density during critical periods and adolescence are well-documented in both mammalian models and in humans, whereby global spine density reductions facilitate the formation of viable connections (Arikkath 2012, Bourne & Harris 2008, Gonzalez-Burgos et al 2015, Knott et al 2006, Poon 2018, Selemon 2013). Compared to the adult cortex, developing networks display higher plasticity during a time window where both environmental and genetic factors can shape the development of cortical circuits (Hensch 2005, Maffei et al 2010, Maffei & Turrigiano 2008). In this Chapter, the density of dendritic spines is increased at P60 - a previously undefined developmental period in the adolescent mouse cortex - prior to pruning at adult P90, a process essential for the fine-tuning of synaptic connections (Boivin et al 2018, Flurkey et al 2007, Johnson et al 2016, Koss et al 2014, Petanjek et al 2011). Knowledge of when these periods of increased capacity for plasticity occur are essential for targeting early stages during which activity-dependent insults may be impacting cortical regions. Though measures of density provide insight into the availability of viable synaptic compartments, they do not capture the structural plasticity of dendritic spines within a network. To

Figure 3.4.1

The schematic represents the relative proportions of dynamic (waved line) and stable (straight line) dendritic spine populations of the motor (A) and somatosensory (B) cortices, within male (red) and female mice experiencing high (green) or baseline (blue) levels of oestrogen. The axis represents the relative proportions of dynamic and stable dendritic spine populations from adolescent P60 to adult P90.

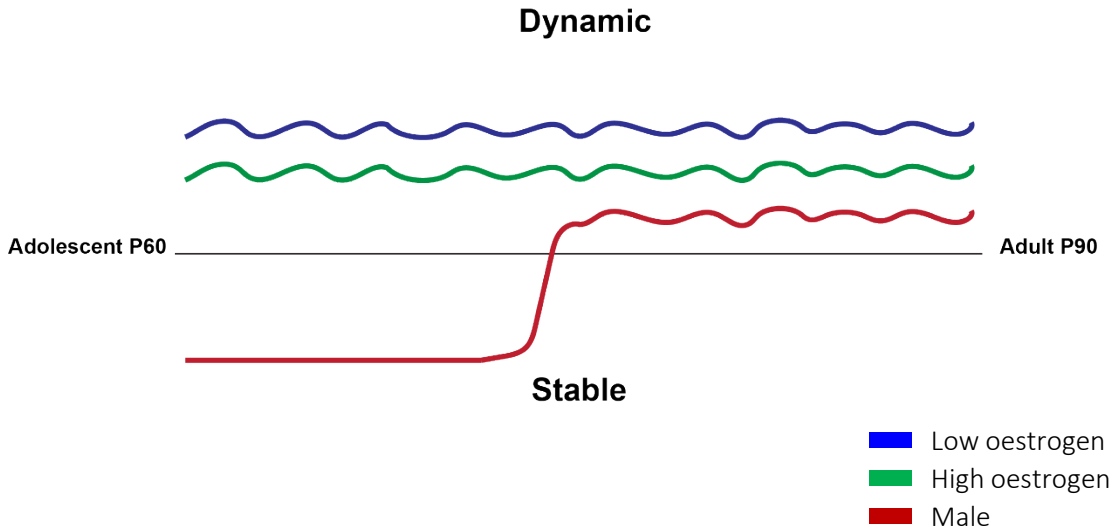
A

Motor cortex



B

Somatosensory cortex



better appreciate the distinct roles of dendritic spines within circuits, the quantification of gain, loss and morphological alterations can be used to further elucidate the neuroplastic processes of a cortical region (Berry & Nedivi 2017).

3.4.2 The male motor cortex maintains high dynamic populations of dendritic spines

The constant rewiring of neural networks is in part mediated through structural plasticity at the dendritic spine, and is a fundamental mechanism for learning. The motor cortex is intricately connected to frontal regions of the brain in mice, and in both mice and humans constitutes a higher order region (Barthas & Kwan 2017, Brecht 2011, Kolb et al 2012, Lein et al 2006, Meunier et al 2009). Accordingly, the primary motor cortex has been implicated in a range of functions outside of typical motor behaviours, and is increasingly recognised as being responsible for higher order tasks such as planning, orientation, angular coordinates during turning tasks and movement anticipation (Brecht 2011, Erlich et al 2011, Hill et al 2011, Sreenivasan et al 2016). The involvement in executive functions as well as motor coordination suggests the region may have higher activity dependent demands, reflected here in the increased turnover of dendritic spines at adolescent P60 and sustained dynamic spine proportions by adulthood (Figure 3.4.1).

Within the male motor cortex, the findings here indicate mature spines undergo higher rates of gain and loss than immature spines. Classically, mature spine types have been considered a stable dendritic spine morphology-however, more recent studies have shown that while spines undergoing dynamic events are likely to be slightly smaller, they can be of all sizes and morphological subtypes (Holtmaat et al 2005, Keck et al 2008, Villa et al 2016). Indeed, increased turnover of spines is the initial step for stabilisation that determines integration into a local circuit (Attardo et al 2015, Hayashi-Takagi et al 2015, Sanders et al 2012). The increased turnover of mature spine types within the motor cortex may be a reflection of increased activity within higher order cortical regions, where mature spines in these regions require more synaptic inputs to stabilise AMPA receptors at the post-synaptic density (Lambert et al 2017, Taft & Turrigiano 2014). Intriguingly, other *in vivo* studies have demonstrated the stabilisation of networks is largely driven by mature or stable spine elimination, implicating the spine type in a range of activity dependent processes underlying circuit formation (Knott & Holtmaat 2008, MacDonald et al 2017). A reduction of thin spine gain in the male motor cortex by adulthood is further indicative of regional maturation, with these spines having the lowest ratio of AMPA to NMDA receptors, and the least capacity for post-synaptic machinery (Holtmaat et al 2005, Majewska et al 2006). These specific alterations in the turnover rates of dendritic spine types may reflect the

maturation of motor cortex circuitry in male mice, with decreases in immature turnover and increases in mature turnover underlying the sustained dynamic nature of the region.

3.4.3 Cycling oestrogen regulates the stable:dynamic ratio of dendritic spines of the *Thy1-YFP* motor cortex at P60

An intriguing result from the current Chapter is how cycling oestrogen regulates the proportion of stable and dynamic dendritic spine populations. During baseline levels of oestrogen, females display similarly high proportions of dynamic spines as males; however, during peaks of oestrogen, dynamic proportions are reduced and dendritic spines within the region are stabilised (Figure 3.41). Oestrogen exerts its effects at the synapse through receptors within the post-synaptic density, with the classical subtypes- alpha and beta- observed to have roles in promoting memory consolidation and spine density that vary depending on cortical region and age (Hara et al 2015, MacLusky et al 2005, Milner et al 2005, Murakami et al 2006, Phan et al 2011, Srivastava et al 2011). Receptors for oestrogen are widely distributed throughout the brain, and the cortex in particular has been shown to undergo increases in dendritic spine density in relation to higher oestrogen levels (Chen et al 2009, Khan et al 2013, Kinsley et al 2006, Tang et al 2014, Wallace et al 2006). Observed increases in dendritic spine density following heightened oestrogen are likely to be associated with the formation and stabilisation of dendritic spines, and the association between high oestrogen and the stabilisation has been suggested to be regulated via the oestrogen receptor β (ERβ) (Alexander et al 2018, Wang et al 2018). While increases in overall dendritic spine density following peaks of oestrogen may reflect the neuroplastic capacity of the cortex, these findings do not provide insights into the strength and stability of synaptic connections being formed and maintained during these stages.

At the level of the dendritic spine, oestrogen increases synaptic proteins, promotes F-actin-dependent spine assembly and activates changes to the ratios of AMPA and NMDA receptors (Babayan & Kramár 2013, Kramár et al 2009, Lee et al 2003, Woolley & McEwen 1994). Previous studies have identified increased binding for specific NMDA receptors at the dendritic spine membrane, with no overall effect on AMPA receptors (Cyr et al 2001, Gureviciene et al 2003, Woolley et al 1997). However, more recent research has found specifically targeting ERβ has profound effects on AMPA recruitment at the spine head, particularly that of GluA1 and GluA2 – essential for activity-dependent delivery of AMPA receptors to the synapse and calcium permeability respectively (Lee et al 2003, Shi et al 2001, Waters et al 2009). It may be that cycling peaks of oestrogen regulate the transient stabilisation of dendritic spines through the ERβ-dependent recruitment of AMPA subunits to the spine head, further inducing F-actin to promote mature spine growth and greater post-synaptic machinery. Further evidence for this

comes from the specific regulation of mature spine gain and loss by oestrogen fluctuations as identified in this Chapter, indicating the hormone is crucial for the formation and regulation of strong synaptic connections.

3.4.4 The somatosensory cortex displays sex-dependent trajectories of maturation

The manipulation of inputs and stimulation differentially affect the rate of spine dynamics in a circuit-specific manner, and reflect region and cell types (Berry & Nedivi 2017). While the motor cortex appears to normally be highly dynamic, with spine stability regulated by oestrogen, the somatosensory cortex in this Chapter displays sex-specific changes at the dendritic spine. Females maintain high dynamics within the somatosensory cortex over time, whilst males display a developmental switch; during adolescence, dendritic spines in the motor cortex of male mice are more stable, whereas by adulthood they become more dynamic (Figure 3.4.1). This dynamic population appears to be driven primarily by the gain of mature spine types-the morphological subset most likely to form stable synaptic connections. Previous studies of dendritic spine turnover within the somatosensory cortex have identified increased stability as mice mature; however, these findings have been at differing time points to the ones utilised in this Chapter (Holtmaat et al 2005; Zuo et al 2005). Further, an increased capacity for turnover has been identified in the somatosensory cortex compared to other cortical regions by adulthood, indicative of a greater plastic capacity (Holtmaat et al 2005). Primarily, these findings come from male mice only, or mixed cohorts; there is less evidence for the effects sex hormones may have over these developmental time periods. Evidence for sex-dependent development of cortical networks has largely come from longitudinal MRI, demonstrating global changes in the size of cortical regions dependent on sex during development; yet the specific events underlying the formation of networks during this time remain largely unknown (Qiu et al 2018, Raznahan et al 2010). The distinct difference in stability vs dynamics between males and females at adolescence within this region may reflect unique timelines for hierarchical cortical development, whereby the somatosensory cortex is evidenced to mature earlier in females than in males (Boivin et al 2018, Munoz-Cueto et al 1990). As aforementioned, the increased dynamic proportions of females over time and that of males by adulthood may reflect the maturing cortex; rather than dynamics being a sign of instability, these populations may instead be contributing to the continual stabilisation of cortical networks through neuroplasticity (Attardo et al 2015, Hayashi-Takagi et al 2015, Lambert et al 2017, Taft & Turrigiano 2014).

As opposed to the motor cortex, where oestrogen exerts differential effects on stable and dynamics spine populations, here dynamic proportions remain elevated through to adulthood regardless of

oestrogen fluctuations. This suggests not only a functionally mature region, but also indicates mechanisms underlying learning, memory and activity-dependent processes remain stable across the oestrus cycle. Evidence for this comes from studies of dendritic spine density on layer V pyramidal neurons of the somatosensory cortex, where steady-state conditions result in dendritic spines being unaffected by oestrus cycle, and comparable to that of males at adulthood (Alexander et al 2018). However, it appears the ability of layer V neurons in this region to respond to sensory-evoked structural plasticity may indeed be influenced by oestrus, displaying higher turnover rates during high oestrus (Alexander et al 2018). Together, these findings indicate that while dendritic spines of the female somatosensory cortex maintain high dynamics over time, circuitry is still regulated by oestrogen cycling during learning events. To continue gaining an understanding of how oestrogen mediates dendritic spine activity, it is vital to investigate how functional output from a region is tied to the machinery at the post-synapse that may interact with or be influenced by the sex hormone.

3.4.5 Conclusions

Dendritic spines are highly motile regulators of the processes underlying neuroplasticity, influenced by a range of factors. The finely-tuned balance of stable and dynamic dendritic spine populations is essential for the appropriate functioning of discrete cortical regions, and underlies the ability to respond to activity demands. This Chapter provides insights into how the neuroplasticity of discrete cortical regions is influenced by age and sex. Within the motor cortex, males and females demonstrate sustained high proportions of dynamic spines, yet females also experience higher stability during peaks of cycling oestrogen. Conversely, within the somatosensory cortex males display a switch whereby adolescence is marked by stable spine populations, whilst by adulthood these populations have become more dynamic. Females display sustained somatosensory dynamic populations from adolescence to adulthood. Gaining a greater understanding of the potential for cortical regions to differentially respond to activity and modulate neuroplasticity is essential for understanding why certain circuits are primed to fail in disease. Here, the increased dynamic needs of the male motor cortex suggest that the region may be specifically susceptible to altered neuroplasticity due to an inability to regulate activity physiologically. It is crucial to investigate the mechanisms underlying interactions between sex, age and region to elucidate how these factors influence neuroplasticity at the dendritic spine both normally and in disease.

4 THE TDP-43^{A315T} MUTATION ALTERS DENDRITIC SPINE DYNAMICS

4.1 INTRODUCTION

Neuroplasticity is the phenomenon describing molecular changes that constitute learning and memory, and is partly regulated by changes in the shape and number of dendritic spines within networks (Fu & Zuo 2011). Changes at the dendritic spine are critical for the appropriate function of cortical regions, and in the current thesis the dendritic spines of the motor cortex have been identified as innately dynamic. However, the region has also been identified as being susceptible to early dysfunction at the postsynaptic structure mediated by misprocessed TDP-43. It is crucial to identify how the normal processes of neuroplasticity may be perturbed in the presence of a disease-linked protein mutation, in order to establish early pathological events that may provide therapeutic targets.

TDP-43 has been implicated in the growth and maturation of dendritic spine through a number of cellular pathways, including the Rho GTPase Rac1 pathway of spinogenesis (Hayashi & Majewska 2005, Luo 2000, Zhang et al 2010). Activity of Rac1 induces AMPAR clustering at the spine head- responsible for strengthening and maturation of synapses- which is facilitated by the trafficking of synaptic mRNAs to and from the synaptic membrane, essential for localised protein synthesis (Grienberger et al 2015, Haditsch et al 2009, Matsuzaki et al 2004, Sutton & Schuman 2006). TDP-43 is a negative regulator of this pathway both *in vitro* and *in vivo*, and changes in protein expression result in aberrant spine formation (Majumder et al 2012). TDP-43 forms a complex with the FMRP protein to regulate the mRNA of both Rac1 and GluA1 proteins, both requiring activity-dependent localised protein synthesis to perform functions at the dendritic spine (Majumder et al 2016). The role of TDP-43 in this pathway may be essential for cortical plasticity under normal conditions, and thus protein misprocessing has vast implications for the ability of susceptible cortical regions to modulate and respond to activity. After establishing changes at the dendritic spine under normal conditions as influenced by age, sex and region, the third aim of this thesis was to investigate dendritic spine plasticity in real time in the *TDP-43^{A315T}* mouse model of ALS.

The sex hormone oestrogen has been implicated in synaptic regulation at the dendritic spine, facilitating fast excitatory postsynaptic potentials, long-term potentiation and increase dendritic spine density (Arevalo et al 2015, Babayan & Kramár 2013, Frankfurt & Luine 2015, Luine & Frankfurt 2013). Intriguingly, oestrogen has also been identified as a potential cortical neuroprotective agent within mammalian models and humans, where decreased hormone levels as a result of ovariectomy or other means increases brain damage under neurodegenerative conditions (Behl 2002, Carswell et al

2005, Scott et al 2012, Tang et al 1996, Zhang et al 2014). Furthermore, the magnitude of the insult is thought to be mediated by fluctuating oestradiol levels during the oestrous cycle (Behl 2002, Berman et al 1997, Erickson et al 2005, Scott et al 2012). In ALS, the incidence of disease is higher among men than women at 4:1 when the age of onset is the second decade, but this rises to a 1:1 ratio at ages above 60 years – when the vast majority of women have undergone menopause and have greatly reduced oestrogen levels (Haverkamp et al 1995, Suzuki et al 2007). Furthermore, women who do develop ALS later in life have often experienced earlier onset of menstruation and menopause; indicative of an involvement of sex hormones in both the onset and progression of the disease (Chiò et al 1991, McCombe & Henderson 2010, Suzuki et al 2007).

The findings from the current thesis have established specific sex-dependent changes at the dendritic spine within the motor cortex, where cycling oestrogen promotes transient stability. Understanding the complex interactions between the synaptic roles for oestrogen within cortical regions, neuroprotective mechanisms, and the progression of ALS may give clues as to what processes modify the disease. To investigate real-time region- and sex-dependent dendritic spine changes in disease states, male and female *Thy1-YFP:prpTDP-43^{A315T}* (*TDP-43^{A351T}*) mice underwent cranial window surgeries at P50. Prior to 2PLSM imaging, the oestrus cycle of female mice was recorded, and these mice then assigned to 'baseline oestrogen levels' or 'high oestrogen levels' cohorts for imaging. The gain, loss, and morphological maturation of dendritic spines was quantified in the motor cortices of *TDP-43^{A315T}* mice, and compared to that of *Thy1-YFPH* controls to establish whether TDP-43 misprocessing or sex influenced the dynamics of dendritic spines within the motor cortex. MATLAB and Neurolucida^{TM} software were utilised for the acquisition and quantification of images. These experimental paradigms will be used to determine the bases for specific susceptibility of the motor cortex to TDP-43 misprocessing at the dendritic spine, and the potential for oestrogen to modulate ALS progression within the motor cortex at the post-synaptic compartment.

4.2 METHODS

4.2.1 Animals

Experimental procedures utilised both male and female mice and were approved by the Animal Ethics Committee of the University of Tasmania, performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Animals were housed in ventilated cages at 20°C on a 12 hour light-dark cycle, with access to food and water *ad libitum*. *Thy1-YFP* transgenic mice [B6.Cg-Tg (Thy1-YFP-H) 16Jrs/J) (Feng et al 2000; Porrero et al 2010)] and TDP-43^{A315T} transgenic mice [B6.Cg-Tg (Prnp-TARDBP*A315T) 95Balo/J (Wegorzewska et al 2009; Herdewyn et al 2014)] were purchased from the Jackson Laboratory and maintained on a C57BL6 background. *Thy1-YFPH* mice express high levels of yellow fluorescent protein in the motor and sensory neurons of cortical layer V (LV) (Feng et al 2000; Porrero et al 2010). The TDP-43^{A315T} mouse demonstrates an approximate 3-fold increase in expression of human TDP-43^{A315T}, in comparison to endogenous TDP-43 levels, driven on the prion promotor (Wegorzewska et al 2009). These mouse lines were intercrossed to generate the *Thy1-YFP::TDP-43^{A315T}* transgenic mice. *Thy1-YFP* transgenic littermates were used as controls.

4.2.2 Genotyping

Mice were genotyped at the time of weaning (28 day post-natal) using a clipping from the top portion of the ear, which was removed and stored at -20°C until DNA extraction was undertaken. DNA was extracted using an Extract-N-Amp Tissue PCR tissue kit (Sigma-Aldrich, Australia) according to manufacturer's instructions and stored at -4°C. TDP-43^{A315T} PCR amplification was performed using an 11 µl reaction containing 50-100 g of DNA (~1µl), and the following primers (GeneWorks, Australia):1µl oIMR 8744 internal positive control forward (5'-CAA ATG TTG CTT GTC TGG TG-3'); 1µl oIMR 8745 internal positive control reverse (5'-GTC AGT CGA GTG CAC AGT TT-3'); 1µl transgene forward (5'-GGA TGA GCT GCG GGA GTT CT-3'); and 1µl transgene reverse (5' TGC CCA TCA TAC CCC AAC TG-3'). Amplification occurred under the following conditions: 94°C for 3 minutes; 94°C for 30 seconds; 60°C for 30 seconds; 72°C for 30 seconds; steps two-four repeated for 30 cycles; 72°C for 30 seconds; 4°C on infinite hold.

Thy1-YFP PCR amplification was performed using a 10 µl reaction containing 50-100 g of DNA (~1µl) and the following primers (GeneWorks, Australia): 1µl oIMR 7338 internal positive control forward (5'-CTA GGC CAC AGA ATT GAA AGA-3'); 1µl oIMR 7339 internal positive control reverse (5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3'); 1µl transgene forward (5'-TCT GAG

TGG CAA AGG ACC TTA G-3'); and 1µl transgene reverse (5'-TGA ACT TGT GGC CGT TTA CG-3'). Amplification occurred under the following conditions: 95°C for 1 minute; 95°C for 15 seconds; 58°C for 15 seconds; 72°C for 30 seconds; steps two-four repeated for 35 cycles; 72°C for 7 minutes; 11°C on infinite hold.

4.2.3 Oestrus cycle tracking

In mice, the oestrus cycle is divided into 4 stages (proestrus, oestrus, metestrus and diestrus) and repeats every 4-5 days unless interrupted by pregnancy, pseudopregnancy or anestrus (Byers et al 2012) (Figure 3.2.1). The oestrus stage in which female mice underwent 2PLSM was assessed in two steps; a preliminary visual observation of the vaginal opening of each mouse, and verification by vaginal cytology. To evaluate oestrus cycle stage visually, mice were restrained and vaginal opening evaluated based on standard criteria (Champlin et al 1973; Byers et al 2012) (Figure 3.2.2 Ai-iv). A vaginal swab was collected using a cotton tipped swab, wetted with room temperature saline. The swab was inserted into the opening of the vagina of the restrained mouse and rolled against the vaginal wall. Cells were transferred to a dry glass slide (Livingstone, Australia) by rolling the swab across the slide. Slides were then air-dried and stained with toluidine blue (0.l25% toluidine blue in 0.l25% acetic acid) (Sigma Aldrich, Australia) for 10 minutes. The slides were then rinsed with water, overlaid with a cover-slip using fluorescent PermaFluor™ mounting medium (Thermo-Fisher Scientific, Australia) and viewed immediately at 200x magnification under bright-field illumination using an Olympus CKX31 Inverted Microscope (Olympus Australia Pty. Ltd., Australia). The stage of oestrus cycle was determined based on the presence or absence of leukocytes, cornified epithelial, and nucleated epithelial cells (Felicio et al 1984) (Figure 3.2.2 Bi-iv).

4.2.4 Cranial window surgeries

An open-skull cranial window was surgically implanted over either the motor or somatosensory cortices of mice at P50, following the protocol previously described by Holtmaat et al. (2009) (Figure 3.2.3). This timeline enables 10 days of recovery prior to imaging at P60, whereupon ~80% of cranial windows were successful. Experimental mice underwent isoflurane (Henry Schein®, USA) induction (4%, ~0.5 litre min^{-1} O_2), were given a sub-cutaneous (s.c.) injection of the long-acting general analgesic Temgesic (buprenorphine 300 mg/ml dosed at 0.1 mg/kg) (Ilium, Troy Laboratories, Australia) and heads shaved to prevent foreign particles entering the surgical site. Mice were then placed into a stereotaxic frame and isoflurane maintained at 1.5-2%, ~0.5 litre min^{-1} O_2. The cranium was cleaned with three rotations of a cotton swab with the disinfectant Microshield® chlorhexidine (Schulke Pty. Ltd., Australia) and after drying, swabbed again with ethanol (Sigma Aldrich, Australia).

Viscotears® liquid eye gel (Novartis Pharmaceuticals, Australia) was applied and the mouse injected s.c. with the local analgesic bupivacaine (bupivacaine hydrochloride 0.5% dosed at 0.025%) (Pfizer Ltd., Australia) and the anti-inflammatory Metacam (meloxicam 5 mg/ml dosed at 1 mg/kg) (Ilium, Troy Laboratories, Australia).

Using scissors, a circular flap of skin (~1 cm^2) was removed from the skull, and the periosteum removed by gently scraping the skull with sharpened forceps. Vetbond™ (cyanoacrylate 3M™, USA) tissue adhesive was applied to the *temporalis* muscle and wound margins to prevent seepage of serosanguinous fluid, sparing the trepanation area. The cortical regions of interest were identified according to bregma, in order to accurately locate the correct coordinates for the motor cortex (-1.0 mm posterior from bregma, -0.8 mm lateral) and the somatosensory cortex (1.5 mm from bregma, 3.0 mm lateral) (Figure 3.2.3 A, B). A circular groove was drilled around the area of interest (~3 mm in diameter) and cortex buffer (125 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM $CaCl_2$, 2 mM $MgSO_4$) regularly applied to avoid heating. A bone island was left intact, dampened with buffer and sharp angle-tipped forceps inserted into the trabecular bone exposed on the side of the groove to expose the dura. Dexapent (dexamethasone sodium phosphate 5 mg/ml dosed at 4 mg/ml) (Ilium, Troy Laboratories, Australia) was topically applied to the surface of the exposed dura to act as an immunomodulator and prevent regrowth of neomembrane (Figure 3.2.3 C). A circular coverglass (4 mm diameter, #1 thickness) was placed over the dura flush with the skull and Loctite® adhesive (Henkel Adhesives, Australia) applied around the window edges to prevent seepage under the coverslip. The cranial window was sealed with dental cement (Paladur, Kulzer Australia Pty. Ltd., Australia), covering the exposed cranium, the wound margins and the lip of the coverglass. A titanium steel bar (Central Science Laboratory, University of Tasmania, Australia) with screw holes was embedded in the dental acrylic over the intact hemisphere for animal stabilisation during imaging sessions (Figure 3.2.3 D).

Post-surgery, the mouse received a 1ml s.c. injection of saline and was placed back into the home cage on a heat pad for ~30 minutes of recovery. The mouse was considered fully recovered after observation of grooming, eating and normal movement and reflexes. Monitoring was undertaken twice a day for 4 days, then once a day for 6 days, prior to the first imaging round.

4.2.5 Two-photon scanning laser microscopy

To image dendritic spine dynamics in real time, a 2PLSM (Scientifica, UK) equipped with a MaiTai laser (Spectra-Physics, NewSpec Pty. Ltd., Australia) and running custom MATLAB® software (MathWorks, Australia) was used over 3 consecutive imaging sessions, 24 hours apart to image the

motor and somatosensory cortices. The MaiTai laser was run at 910 nm by a 10 W solid state laser (Spectra-Physics, Australia). The mouse was anaesthetised following an intraperitoneal (i.p.) injection of a 12:1 mixture of Ketamine (ketamil 100 mg/ml) (Ilium, Troy Laboratories, Australia) and Xylazine (xylazil 20 mg/ml) (Ilium, Troy Laboratories, Australia) at a recommended dosage of 5 µl of working solution per gram (0.12 mg/g ketamine to 0.01 mg/g xylazine, approximate dose of 100 µl for a 20 g mouse), which wore off after 45-60 minutes. Once no reflexes were detected, mice were placed on a heat-pad maintained at 37°C under the microscope, with head immobilised by screwing the titanium bar of the cap to a stable, adjustable arm and eye gel applied.

An EC Plan-Neofluar 10x/0.3 air objective (Nikon, USA) was used to image the vasculature of a region of interest (ROI), illuminated by white light, and captured using XCAP Image Processing Software (EPIX Incorporated, USA) (Figure 3.2.4 Ai). Once a region of interest was identified, the z-plane coordinate was saved and the objective changed to a W Plan-Apo 40x/1.0 water immersion objective (Nikon, USA). The same z-plane coordinate was initiated and a higher-powered region of interest captured to find the same position during consecutive imaging sessions (Figure 3.2.4 Aii). The MaiTai laser was used to capture blue/green emissions (Channel 1 PMT 550-565ηm) at 910 nm/1700 mW of power at a maximum percentage of 25%, equivalent to 70 mW (Mainen et al 1999). The apical tuft dendrites of LV neurons were captured in Z-stacked images (20µm) of cortical layers II-III (slices 1µm apart) at a minimum depth of 210 µm from the cranial surface to a maximum depth of 350 µm (Figure 3.2.4 B). Landmark dendrites were identified during the initial imaging session, and returned to in the following sessions in order to capture the same region of interest and identify the same dendritic spines on a given dendritic segment (Figure 3.2.4 C). The xyz-coordinates of every ROI were stored relative to the vasculature pattern initially captured. To verify all structures were detected, excitation was increased to ensure no additional spines were revealed and signal fluorescence intensity was different from noise. Imaging sessions lasted for ~30 minutes p/mouse and during imaging the respiratory rate of the mouse was monitored at 10 minute intervals. Post-imaging the mouse was injected with 1ml of saline s.c. and placed back into the transfer cage on a heat pad for recovery, until fully ambulant.

4.2.6 Quantification of dendritic spine turnover

Z-stacked images were taken in real time of LV apical tuft dendrites residing in LII/III of the motor and somatosensory cortices at 3 times points, 24 hours apart (Figure 3.2.3 A, B). To quantify the turnover of dendritic spines, images for a given segment of dendrite from each round were opened in Neurolucida™ and spines annotated along the segment as being morphologically thin, stubby or mushroom shaped. The analysed segments were uploaded into Neurolucida Explorer™ and density

quantified to validate representation of a given cohort. For each animal, ~2 dendrites were quantified for each cohort; P60 MC males (369 spines), baseline oestrus (406 spines) and high oestrus (350 spines), and P60 TDP-43 males (361), baseline oestrus (384) and high oestrus (272). Spines were only measured if they clearly emanated laterally from the dendritic shaft-those protruding below or above the dendrite could not be reliably quantified in the axial dimension. Spines for each round were quantified in comparison to the preceding round as being gained, lost or exhibiting a morphological change-with morphological subtypes being classed as mature (mushroom and stubby morphologies) and immature (thin morphology). Spines were considered lost if they disappeared between imaging rounds; gained if they newly protruded between rounds; and considered to exhibit morphological changes if the head to neck ratio was clearly altered between rounds (Figure 3.2.3 C). The baseline turnover gain and loss rates of spine subtypes was calculated as $TR = N_t/N_l$ or N_g, where TR is the turnover rate; N_t is the total number of spines for a single mouse; N_l is the number of spines lost; and N_g is the number of spines gained, as a ratio from the total number of dendritic spines and dendritic spine changes. This formula was used to calculate the total gain and loss rate of all spines for a single mouse, as well as the specific gain and loss rates of mature, and of immature spines-where N_lm and N_gm is equal to the number of lost and gained mature spines, and N_li and N_gi is equal to the number of lost and gained immature spines (Attardo et al 2015, Frank et al 2018).

4.2.7 Statistical analyses

Student's t-tests, one-way and two-way analysis of variance (ANOVAs) were performed in GraphPad (USA) Prism. Post-hoc comparisons were performed using Bonferroni's correction for multiple comparisons. A p-value of <0.05 was considered significant.

4.3 RESULTS

4.3.1 The male *TDP-43^{A315T}* motor cortex displays altered proportions of stable and dynamic dendritic spine populations compared to *Thy1-YFP* controls at P60

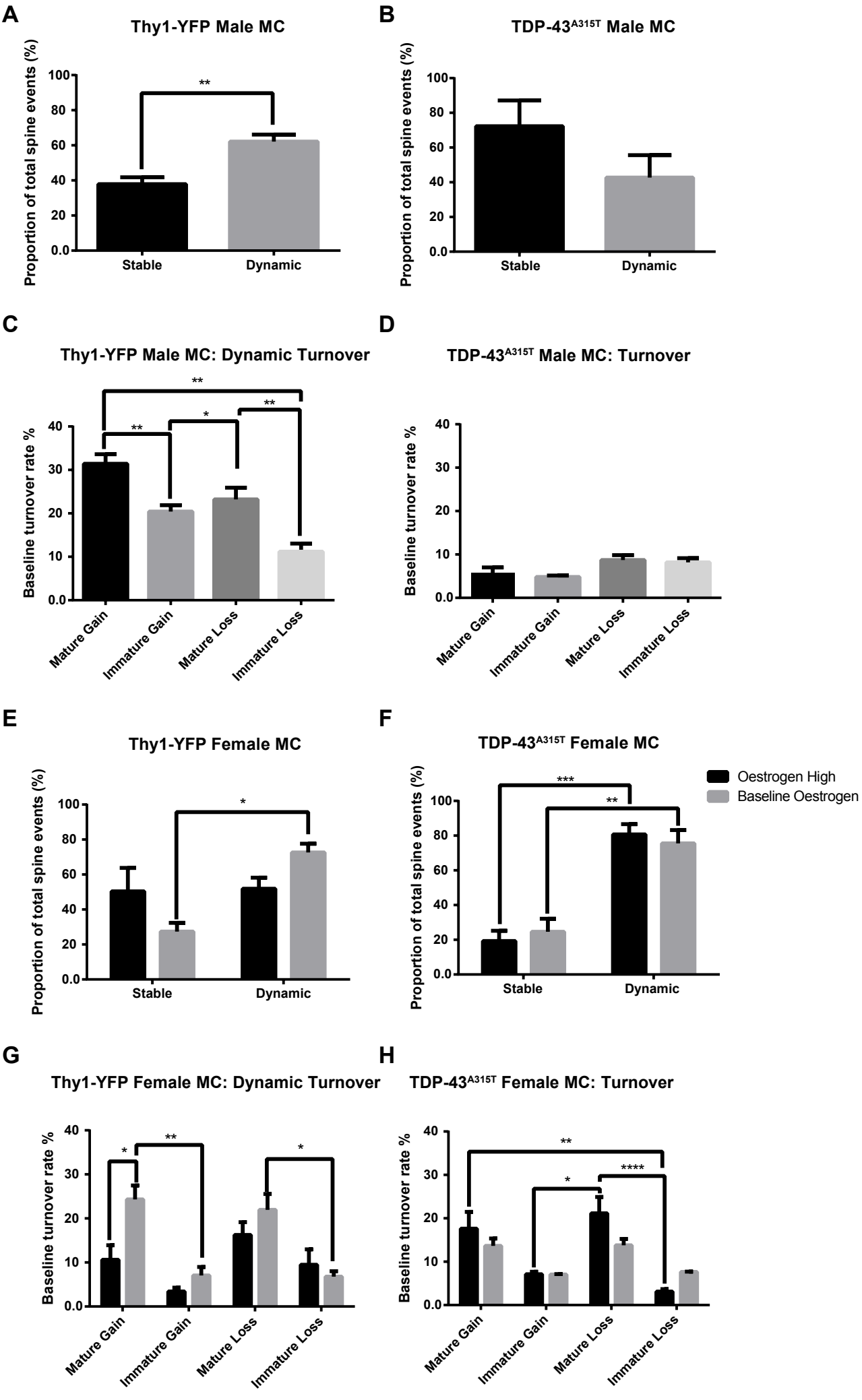
The structure and localisation of dendritic spines are carefully regulated, and abnormalities are a hallmark of several neurological and neurodegenerative disorders (Fiala et al 2002). To investigate the potential for misprocessed TDP-43 to pathologically alter dendritic spine dynamics in ALS, individual apical tuft dendrites of layer V pyramidal neurons residing in layer II/III were followed over three consecutive days, in the motor (MC) cortices of TDP-43^{A315T} male (M) (n = 6) and female (F) (n = 7) mice at P60. To establish the overall spine activity within different cortical regions between the sexes, the total proportion of stable and dynamic spines was quantified within each cohort. Dynamic populations were classified as mature or immature spine types, with a new spine considered 'gained' and the absence of a spine in a subsequent imaging round considered 'lost'.

In the male motor cortex at P60, *TDP-43^{A315T}* mice did not display a significant difference between stable and dynamic populations of dendritic spines compared to controls, nor were there any significant differences within dynamic populations of mature or immature gain or loss turnover rates (Figure 4.3.1 B). However, within the female motor cortex there was a significantly ($p < 0.05$, two-way ANOVA, Bonferroni post-hoc) higher proportion of dynamic spine populations than stable during both high and baseline oestrogen stages (19.30% ± 0.10 F high MC stable, 80.70% ± 0.10 F high MC dynamic, 24.60% ± 0.13 F baseline MC stable, 75.60% ± 0.13 F baseline MC dynamic) (Figure 4.3.1 F). Within the dynamic proportion of dendritic spines of the *TDP-43 ^{A315T}* female motor cortex during high oestrogen, the loss of mature spine types was significantly ($p < 0.05$, two-way ANOVA, Bonferroni post-hoc) greater than the overall turnover of immature spines (21.2% ± 0.07 F high MC mature loss, 7.00% ± 0.01 F high MC immature gain, 3.00% ± 0.01 F high MC immature loss) while the gain of mature spines was significantly greater than immature loss (17.6% ± 0.08 F high MC mature gain) (Figure 4.3.1 H). There was no significant difference between mature gain and immature gain. These results indicate the male *TDP-43^{A315T}* does not display dendritic spine changes between stable and dynamic populations, whilst females display an overall dynamic motor cortex independent of oestrogen levels.

4.3.2 Dynamic dendritic spine changes are increased in the female *TDP-43^{A315T}* motor cortex during high oestrogen at P60

Figure 4.3.1

(A, C, E, G) The graphs show the stable and dynamic proportions of dendritic spines, and the turnover of dynamic spine populations of the motor cortex, in *Thy1-YFP* male and females experiencing high and baseline levels of oestrogen as presented in the previous chapter. (B, D, F, H) Comparisons of stable and dynamic populations of dendritic spines within the *TDP-43*^{A315T} male (n = 5) and female motor cortices at P60. Stable spines were quantified as those unchanged over the three imaging rounds, while dynamic spines underwent a gain, loss or morphological change event from the first imaging round to the last. (B) The graph shows the proportion of stable and dynamic dendritic spines within the male motor cortex. (D) The graph shows the turnover rate of mature and immature spines within the male motor cortex. (F) The graph shows the proportion of stable and dynamic dendritic spines within the female motor cortex during high (n = 3) and baseline (n = 3) levels of oestrogen. (H) The graph shows the turnover rate of mature and immature spines within the female motor cortex during high and baseline levels of oestrogen. MC = motor cortex. Bars show mean ± SEM. *p = <0.05.


A
Thy1-YFP Male MC
Proportion of total spine events (%)
Stable
Dynamic
B
TDP-43A315T Male MC
C
Thy1-YFP Male MC: Dynamic Turnover
Baseline turnover rate %
Mature Gain
Immature Gain
Mature Loss
Immature Loss
D
TDP-43A315T Male MC: Turnover
E
Thy1-YFP Female MC
F
TDP-43A315T Female MC
Oestrogen High
Baseline Oestrogen
G
Thy1-YFP Female MC: Dynamic Turnover
H
TDP-43A315T Female MC: Turnover

As demonstrated in the previous chapter, sex has an influential role on the neuroplasticity of specific cortical regions. Converging research indicates a neuroprotective role for oestrogen in a range of experimental models of cognitive disorders and brain injury (Li et al 2014, Prokai & Simpkins 2007, Sohrabji 2015, Zhang et al 2014). To establish the influence of oestrogen on neuroplasticity at the dendritic spine in the *TDP-43^{A315T}* motor cortex at P60, female mice were imaged at diestrus/metestrus (baseline oestrogen) (n = 4) or proestrus/estrus (high oestrogen) (n = 4). The relative proportions of stable and dynamic dendritic spines were quantified, and changes occurring within the dynamic population were measured and compared between males and females. The male TDP-43^{A315T} motor cortex at P60 had a significantly ($p < 0.05$, one-way ANOVA, Tukey's multiple comparisons test) greater proportion of stable spines compared to females experiencing high oestrogen levels (19.30% ± 0.24 F high MC TDP stable, 72.30% ± 0.53 M MC TDP stable) with no other significant differences in overall stable populations, nor in dynamic populations (Figure 4.3.2 B, D).

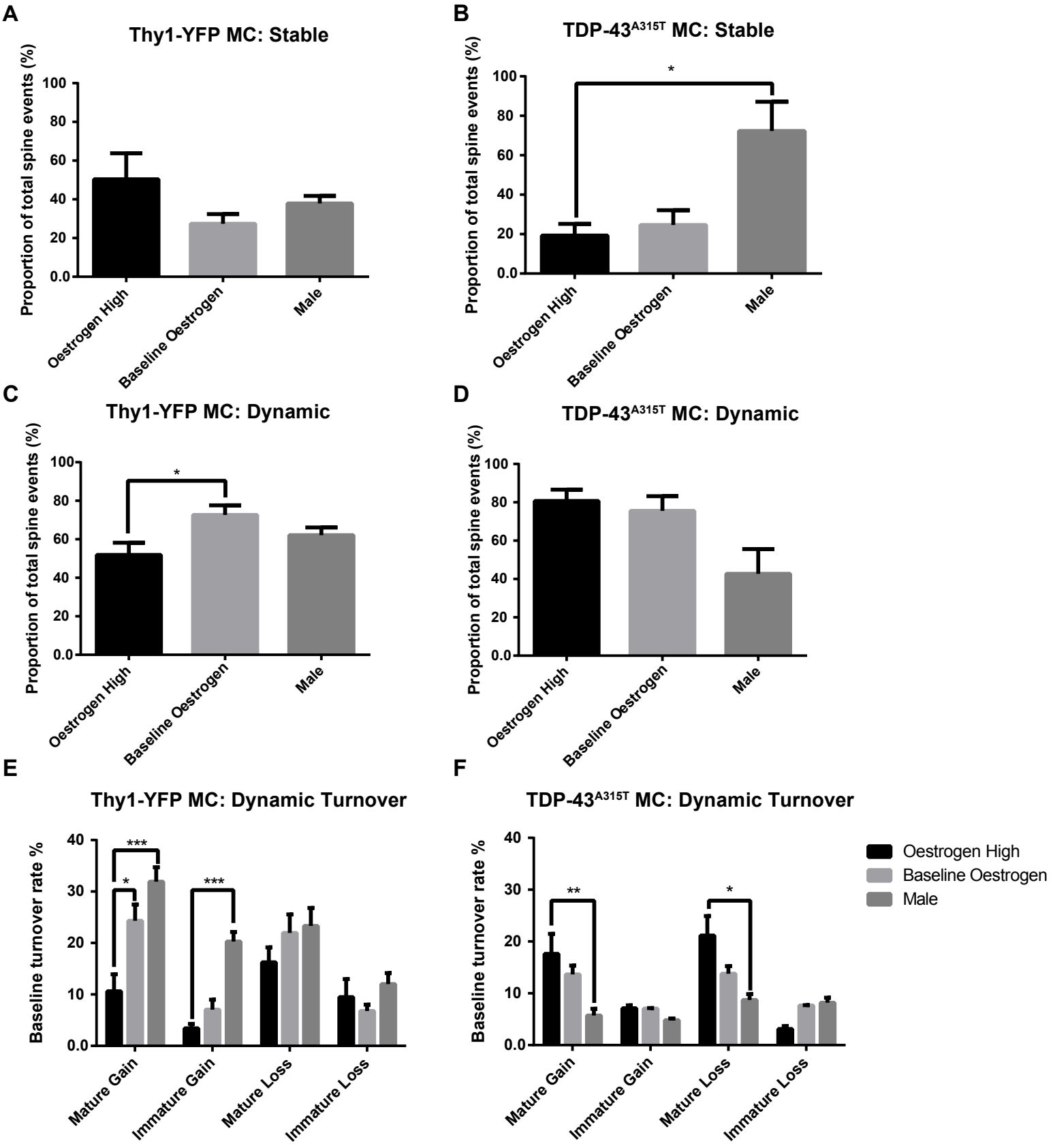
Within the dynamic proportion of dendritic spines, females experiencing high oestrogen levels displayed significantly ($p < 0.05$, two-way ANOVA, Tukey's multiple comparisons test) increased gain and loss rates of mature spine types compared to males (17.60 ± 0.07 F high MC TDP mature gain, 5.70% ± 0.02 M MC TDP mature gain, 21.20% ± 0.07 F high MC TDP mature loss, 8.70% ± 0.02 M MC TDP mature loss). No other significant differences in turnover rates of dynamic spines were identified between males and females (Figure 4.3.3 F). Together, these results suggest high oestrogen levels are mediating transiently increased rates of mature dendritic spine turnover in the TDP-43^{A315T} motor cortex, compared to the relative stability of the TDP-4343^{A315T} male motor cortex.

4.3.3 Dendritic spine dynamics are reduced in the male *TDP-43^{A315T}* motor cortex compared to *Thy1-YFP* mice at P60

To quantify changes to TDP-43-mediated dendritic spine changes at P60, the stable and dynamic proportions of dendritic spines in the male and female *TDP-43^{A315T}* motor cortex were compared to those of the *Thy1-YFP* control cortex (Figure 4.3.3). Male *TDP-43^{A315T}* mice displayed significantly ($p < 0.05$, unpaired t-test) increased proportions of stable spines compared to male *Thy1-YFP* mice within the motor cortex (37.84% ± 0.09 M MC control stable, 72.30% ± 0.30 M MC TDP-43 stable) (Figure 4.3.3 A), with no significant differences between dynamic proportions (Figure 4.3.3 B). Within the dynamic proportion of spines, male TDP-43^{A315T} mice displayed significantly ($p < 0.001$, two-way ANOVA, Tukey's multiple comparisons test) reduced rates of mature gain (31.40% ± 0.50 M MC control mature gain, 5.70% ± 0.02 M MC TDP-43 mature gain), mature loss (23.20% ± 0.06 M MC control mature loss, 8.70% ± 0.02 M MC TDP-43 mature loss), and immature gain (20.40% ± 0.03 M

Figure 4.3.2

Comparisons of stable and dynamic populations of dendritic spines between male and female *TDP-43^{A315T}* motor cortex at P60. (A, C, E) The graphs show the stable and dynamic proportions of dendritic spines, and the turnover of dynamic spine populations of the motor cortex, between *Thy1-YFP* male and females experiencing high and baseline levels of oestrogen for a visual comparison to dendritic spines of the *TDP-43^{A315T}* motor cortex. (B) The graph shows the proportion of stable dendritic spines within the motor cortex of male (n = 5) mice and female mice experiencing high (n = 3) and baseline (n = 3) levels of oestrogen. (D) The graph shows the proportion of dynamic dendritic spines within the motor cortex of male and female mice experiencing high and baseline levels of oestrogen. (F) The graph shows the turnover of mature and immature spines within the motor and somatosensory cortices male mice and female mice experiencing high and baseline levels of oestrogen. MC = motor cortex. Bars show mean ± SEM. *p = <0.05.


A
Thy1-YFP MC: Stable
Proportion of total spine events (%)
100
80
60
40
20
0.0
Oestrogen High
Baseline Oestrogen
Male
B
TDP-43A315T MC: Stable
*
Proportion of total spine events (%)
Oestrogen High
Baseline Oestrogen
Male
C
Thy1-YFP MC: Dynamic
*
Proportion of total spine events (%)
Oestrogen High
Baseline Oestrogen
Male
D
TDP-43A315T MC: Dynamic
Proportion of total spine events (%)
Oestrogen High
Baseline Oestrogen
Male
E
Thy1-YFP MC: Dynamic Turnover
Baseline turnover rate %
40
30
20
10
0.0
***
*
***
Mature Gain
Immature Gain
Mature Loss
Immature Loss
F
TDP-43A315T MC: Dynamic Turnover
Baseline turnover rate %
**
*
Mature Gain
Immature Gain
Mature Loss
Immature Loss
Oestrogen High
Baseline Oestrogen
Male

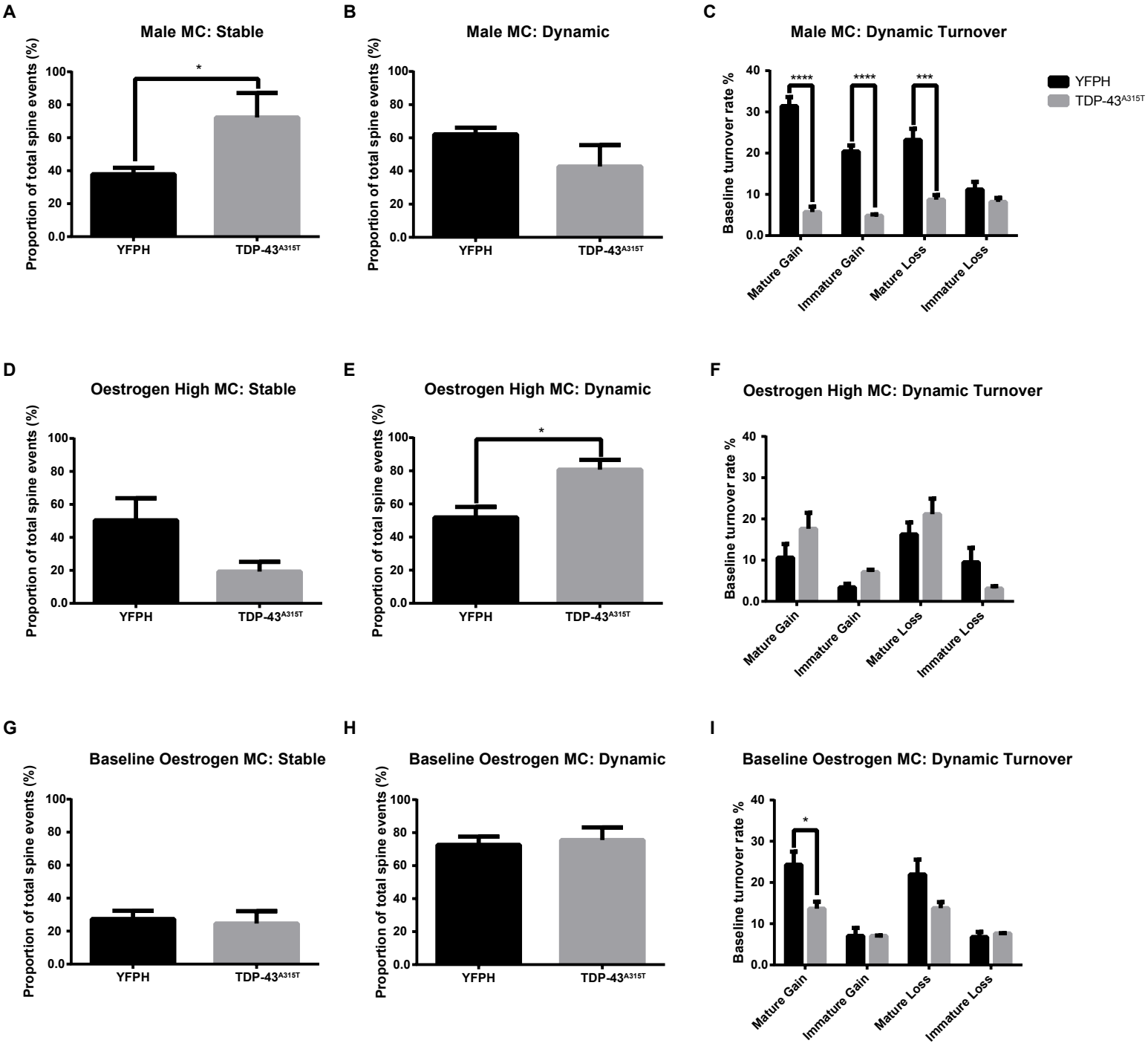
MC control immature gain, 4.80% ± 0.01 M MC TDP-43 immature gain) as compared to male *Thy1-YFP* mice (Figure 4.3.3 C).

4.3.4 Oestrogen peaks rescue the dynamics of the motor cortex in the female *TDP-43^{A315T}* motor cortex compared to controls at P60

Oestrogen has varied pathways through which it exerts neuroprotective influences, and hormone levels mediate functions in cognition and recovery from insult. To investigate the potential for oestrogen to interact with neuroplasticity at the dendritic spine in the presence of TDP-43 misprocessing, the stable and dynamic proportions of dendritic spines within female *TDP-43^{A315T}* mice experiencing baseline or high levels of oestrogen were compared to *Thy1-YFP* counterparts. No significant differences were identified in the proportion of stable spines during baseline or high levels of oestrogen, nor in the proportion of dynamics spines during baseline oestrogen levels (Figure 4.3.3 D, G, H). However, the dynamic proportion of spines was significantly ($p < 0.02$, unpaired t-test) increased during high oestrogen in *TDP-43^{A315T}* female mice population compared to *Thy1-YFP* females (51.91% ± 0.80 F high MC control dynamic, 80.71% ± 0.10 F high MC TDP-43 dynamic) (Figure 4.3.3 E). Within the dynamic dendritic spine proportion, no differences in turnover rates were identified during high oestrogen levels (Figure 4.3.3 F); however, *TDP-43^{A315T}* mice experiencing baseline oestrogen levels displayed a significant ($p < 0.0.5$, two-way ANOVA, Tukey's multiple comparisons test) reduction in the rate of mature spine gain compared to *Thy1-YFP* females at P60 (24.30% ± 0.06 F baseline MC control mature gain, 13.70% ± 0.03 F baseline MC TDP-43 mature gain) (Figure 4.3.3 I). The data indicates transient peaks of oestrogen may be directly associated with increased dynamics of the female *TDP-43^{A315T}* motor cortex, compensating for TDP-43-mediated dysfunction at the synapse.

Figure 4.3.3

Comparisons of stable and dynamic populations of dendritic spines within the male and female *Thy1-YFP* and *TDP-43^{A315T}* motor cortices. (A, D, G) The graphs show the stable proportions of dendritic spines between *Thy1-YFP* and *TDP-43^{A315T}* motor cortices within males (A), females experiencing high oestrogen (D) and females experiencing baseline oestrogen (G). (B, E, H) The graphs show the dynamic proportions of dendritic spines between *Thy1-YFP* and *TDP-43^{A315T}* motor cortices within males (B), females experiencing high oestrogen (E) and females experiencing baseline oestrogen (H). (C, F, I) The graphs show the gain and loss of mature and immature spine types within dynamic dendritic spine proportions between *Thy1-YFP* and *TDP-43^{A315T}* motor cortices within males (C), females experiencing high oestrogen (F) and females experiencing baseline oestrogen (I). MC = motor cortex. Bars show mean ± SEM. *p = <0.05.


A
Male MC: Stable
Proportion of total spine events (%)
100
80
60
40
20
0.0
*
YFPH
TDP-43A315T
B
Male MC: Dynamic
Proportion of total spine events (%)
YFPH
TDP-43A315T
C
Male MC: Dynamic Turnover
Baseline turnover rate %
40
30
20
10
0.0
****
****
***
YFPH
TDP-43A315T
Mature Gain
Immature Gain
Mature Loss
Immature Loss
D
Oestrogen High MC: Stable
Proportion of total spine events (%)
YFPH
TDP-43A315T
E
Oestrogen High MC: Dynamic
Proportion of total spine events (%)
*
YFPH
TDP-43A315T
F
Oestrogen High MC: Dynamic Turnover
Baseline turnover rate %
Mature Gain
Immature Gain
Mature Loss
Immature Loss
G
Baseline Oestrogen MC: Stable
Proportion of total spine events (%)
YFPH
TDP-43A315T
H
Baseline Oestrogen MC: Dynamic
Proportion of total spine events (%)
YFPH
TDP-43A315T
I
Baseline Oestrogen MC: Dynamic Turnover
Baseline turnover rate %
*
Mature Gain
Immature Gain
Mature Loss
Immature Loss

4.4 DISCUSSION

Disturbances in the structure and function of dendritic spines have been associated with the onset and progression of a number of neurodevelopmental, psychiatric, and degenerative disorders; though when and how these changes impact disease has yet to be elucidated. Dendritic spines are a key regulator of neuroplasticity, and their viable function is crucial to the ability of cortical networks to respond to and modulate activity. Changes at the dendritic spine are influenced by the cortical region in which they reside, age and sex, and it is essential to understand how these factors may influence the structure in both normal conditions and in disease states. The findings of this thesis implicate dysregulated neuroplasticity at the dendritic spine as being an early disease event, specifically affecting the motor cortex in a model of ALS. In addition, the observation of increased neuroplasticity during high oestrogen in the presence of a TDP-43 mutation suggests the sex hormone may be a viable treatment pathway to restore dendritic spine function. The current chapter investigated dendritic spine plasticity in real time in the TDP-43^{A315T} mouse model of ALS, to ascertain how early synaptic dysfunction may impact neuroplasticity at the postsynaptic structure.

4.4.1 The heightened dendritic spine turnover of the motor cortex may confer select vulnerability in the presence of the *TDP-43^{A315T}* mutation

A reduction in the normal high dynamics of the male motor cortex was identified in the presence of TDP-43 misprocessing at a presymptomatic time point. The motor cortex represents a higher order cortical region in the mammalian brain, including humans, featuring immense storage capability and modifiable networks controlled by broad activity patterns within local, intercortical and subcortical circuits (Auer et al 2018, Duncan & Owen 2000, Sanes & Donoghue 1997). The region has been shown to rapidly establish dynamic and adaptive synaptic connections in response to activity, which may be indicative of a greater need for dynamic synaptic reorganisation to respond to inputs and modulate large circuits (Chen et al 1998, Ziemann et al 1998). Accordingly, the motor cortex is involved in activity beyond roles strictly associated with upper motor neuron function and is activated during executive functions and cognition, with high neuroplastic demands (Kukleta et al 2016, Wexler et al 1997). This need for dynamic synaptic plasticity may result in an increased susceptibility to activity-dependent insult in diseases such as ALS.

Pathological alterations in ALS are thought to occur long before symptom manifestation, yet thus far interactions between synaptic plasticity and protein aggregation have only been associated with disease onset (Arendt et al 1998, Picconi et al 2012, Stephan et al 2002, Tampellini et al 2009). Here, TDP-43-mediated changes in neuroplasticity at the dendritic spine are evident presymptomatically in the

male motor cortex. Furthermore, this presymptomatic time point has been identified as being a period of increased spine density, indicative of a greater capacity for neuroplasticity. During these periods, cortical regions are especially susceptible to extrinsic insults; the higher activity demands of the motor cortex at this time may thus render the region innately vulnerable to dysfunction at the synapse (Purves et al 1988, Schaefers & Teuchert-Noodt 2016).

It is still unclear as to whether the reduction in dendritic spine dynamics as identified in the current Chapter is due to a loss of connectivity, or to compensation of over-activity within the network. A range of hypotheses have been proposed in regards to network-related neurodegeneration, and these include nodal stress, where regions undergo activity-related traffic inducing 'wear and tear'; trophic failure, where disruption of normal connectivity undermines neuronal support; and shared vulnerability, where regions featuring a specific pattern of protein expression exhibit evenly distributed susceptibility throughout the network (Appel 1981, Buckner et al 2009, Salehi et al 2006, Saxena & Caroni 2011). With evidence for early alterations to neuroplasticity mediated by TDP-43, it is essential to understand how other factors may be influencing synaptic function at the dendritic spine in disease states within the motor cortex.

4.4.2 High oestrogen is associated with high dendritic spine dynamics in the presence of the *TDP-43^{A315T}* mutation

Compared to the decreased dendritic spine dynamics of the male *TDP-43^{A315T}* motor cortex, females display an overall heightened dynamic population of dendritic spines within this region. Additionally, cycling peaks of high oestrogen are associated with heightened dynamics in the presence of *TDP-43^{A315T}* as opposed to greater stability in control mice. This may be associated with compensatory mechanisms, whereby in disease states synaptic alterations have been shown to initially compensate for activity-dependent insult by rapidly reorganising synaptic connections- yet how dendritic spines respond during these conditions has not been established (Enciu et al 2011, Schaefers & Teuchert-Noodt 2016, Zhou et al 2012). The increased dynamics of dendritic spines during high oestrogen in *TDP-43^{A315T}* females may therefore be due to triggering neuroprotective processes.

Oestrogen-mediated protection has been well documented *in vitro* and *in vivo*, though the mechanisms involved are widely unknown. Treatment with oestrogen agonists have demonstrated diverse mechanisms of potential neuroprotection, particularly through modulation of neuroplasticity in Parkinson's, Alzheimer's disease, ischemia and Multiple Sclerosis (Brann et al 2007, Cho et al 2003, Garcia-Segura et al 2001, Gold & Voskuhl 2009). Evidence for the pathways through which oestrogen may exert neuroprotective effects come from the normal localisation of the enzyme aromatase at the

synapse (Balthazart et al 1991, Garcia-Segura 2008, Remage-Healey et al 2011, Zhou et al 2014). Aromatase is responsible for the biosynthesis of oestrogen, and is now thought be a key synaptic protein in human and mouse tissue. Accordingly, the levels of protein identified are dependent on the amount of stimulation at the synaptic compartment (Zhou et al 2014). This provides a direct link between stimulation and oestrogen production at the postsynapse, and indicates that neuroprotective processes may arise from activity-dependent mechanisms. It is important to note that the timing of oestrogen administration is critical for a protective outcome, whereby if used too late in disease it no longer confers protective effects (Brinton 2008, Nilsen 2008, Scott et al 2012). An additional caveat to the use of oestrogen to potentially restimulate damaged neuronal networks is the unknown actions of receptor subtypes during neuroprotection. While oestrogen receptor alpha (ERα) has been most commonly linked to neuroprotection during ischemia and injury of CA1 hippocampal neurons, the overall application of oestrogen is protective against kainate and NMDA neurotoxicity, and accumulating research now implicates both receptors in activity-dependent neuroprotection (Bi et al 2000, Cordey & Pike 2005, Wnuk et al 2012).

With evidence here for the ability of oestrogen to potentially enhance the dynamics of the motor cortex in disease as a possible method of compensation, the exact time at which application of an agonist may be most effective, as well as which receptors require stimulation has yet to be elucidated. For any therapeutic treatments utilising oestrogen to be effective in promoting neuroplasticity, these factors need to be accounted for to optimise maintenance of normal dendritic spine activity and synaptic connections within vulnerable regions.

4.4.3 Mature spines are selectively altered in the presence of the *TDP-43^{A315T}* mutation

In the current Chapter, mature spine turnover rates within the male *TDP-43^{A315T}* motor cortex are found to be severely reduced. Furthermore, *TDP-43^{A315T}* females experiencing high oestrogen display an increase in mature spine gain, whilst females experiencing baseline oestrogen levels display a reduction in normal mature spine gain. Together, these findings suggest mature spine types may be most affected in the presence of TDP-43 misprocessing in a model of ALS. Mature spine types are typically recognised through their larger size and a greater ratio between the spine head and neck, creating a ‘mushroom’ morphology featuring a high number of postsynaptic receptors (Holtmaat et al 2005). Synaptic transmission and neuronal activity play key roles in sculpting neuronal circuits by regulating maturation of dendritic spines, and excitatory glutamatergic transmissions are particularly key to this through actions at AMPA and NMDA receptors at the dendritic spine head (Lai & Ip 2013, McKinney 2010, Ultanir et al 2007). The number of AMPARs in particular has been associated with

synaptic strength and the size of the dendritic spine (Anggono & Huganir 2012, Matsuzaki et al 2001, Miyazaki & Ross 2017). Localised protein synthesis has also been implicated in modulating dendritic spine morphology in an activity-dependent manner, and the trafficking of synaptic mRNAs to the postsynapse following activation is critical for neuroplasticity (Fernandez-Moya et al 2014, Mitsumori et al 2017, Sutton & Schuman 2006, Swanger & Bassell 2013).

TDP-43 has been implicated in dendritic spine maturation through the Rac1 pathway of spinogenesis, associated with AMPAR insertion at the spine head. Furthermore, TDP-43 forms a translational complex with the FMRP protein to process the AMPA subunit GluA1 (Ederle & Dormann 2017, Majumder et al 2012, Majumder et al 2016, Udagawa et al 2015). The GluA1 subunit has been termed the 'gatekeeper of neuroplasticity' due to the phosphorylation of the protein being strongly associated with LTP and the role for the subunit in the recruitment of other AMPA subunits following activity (Diering & Huganir 2018, Henley & Wilkinson 2013). In the presence of TDP-43 misprocessing, it may be that a reduced ability for the protein to traffic and process synaptic mRNAs such as GluA1 at the dendritic spine renders the structure unable to mature in response to activity and localised protein synthesis. This may then have broader effects on the ability of appropriate synaptic connections to be formed and maintained, particularly in the case of dendritic spine maturation. In the current thesis, this is particularly salient for the findings here within the motor cortex, whereby the dynamic turnover of mature spine types is severely reduced in male *TDP-43^{A315T}* mice, as well as in females with baseline levels of oestrogen.

4.4.4 Conclusions

The results here elucidate a previously unknown early disease event occuring in the *TDP-43^{A315T}* mouse model of ALS, whereby neuroplasticity at the dendritic spine is selectively perturbed in the motor cortex. Specifically, males display a reduced ability to maintain dynamic populations of dendritic spines in the motor cortex, in comparison to the increased dynamics of the region under normal conditions. Furthermore, this Chapter indicates the sex hormone oestrogen may be acting to compensate for lost dendritic spine dynamics within the female *TDP-43^{A315T}*; normally associated with stability, instead cycling peaks of oestrogen maintain increased dynamic populations of dendritic spines within the motor cortex. Together, these findings suggest that the male motor cortex is inherently susceptible to activity-dependent insult at the dendritic spine as mediate by misprocessed TDP-43, and that this could potentially be protected by the appropriate administration of oestrogen. Further studies are required to resolve the mechanism through which TDP-43 may be mediating dendritic spine dysfunction, as well as the specific receptor subsets of oestrogen that convey

neuroprotective effects. Collectively, these findings support the hypotheses that TDP-43 misprocessing affects the dynamics of dendritic spine turnover and perturbs neuronal plasticity at an early time point.

5 GENERAL DISCUSSION

The preclinical progression of neurodegenerative disease has received increasing attention as one of the major obstacles to developing disease-modifying therapies. It is evident that identifying pathological pathways that occur prior to disease manifestation is key to developing viable therapeutic targets. One such pathway that has been increasingly identified in a range of neurodegenerative disorders is that of early synaptic dysfunction, whereby perturbations to synaptic structure and functions precede cell death and overt disease symptoms (Bae & Kim 2017, Lepeta et al 2016, Wishart et al 2006). ALS features the select vulnerability of the motor system to progressive neuronal loss, and is characterised by toxic inclusions within the neuronal cytoplasm (Neumann et al 2006). The RNA-binding protein TDP-43 has been identified as the primary component of these aggregates in the vast majority of disease cases; whilst classically, research has focused on the impact of TDP-43 misprocessing in the nucleus and the cytoplasm, it is now thought to have more distal roles at the synaptic compartment. Early dysfunction at the synapse has been increasingly linked to protein dysfunction in ALS, and evidence of normal functions for TDP-43 at the synapse suggest the potential for misprocessing to be a pathological pathway in disease.

Dendritic spines are the primary postsynaptic compartment of glutamatergic neurons and are integral to the processes underlying neuroplasticity; encompassing the connectivity of neural circuits and the ability of the cortex to reorganise and modulate activity in response to stimulation such as learning events (Bosch & Hayashi 2012, Frankfurt & Luine 2015, Woolfrey & Srivastava 2016). Highly motile structures, their morphology is tightly linked to functions that dictate the strength of synaptic connections (Holtmaat et al 2005). Disease-specific disruptions to dendritic spine shape, size or number accompany a range of neurological diseases and disorders within discrete neuroanatomical regions, suggesting they may be a common substrate for the select vulnerability of cortical regions. TDP-43 has been observed to have normal roles at the dendritic spine, yet it has remained unknown as to how the structure may be impacted in disease states such as ALS, and why specific cortical regions are so susceptible to pathology at the synapse. The current thesis demonstrates that the misprocessing of TDP-43 has implications for the structure and function of the dendritic spine, indicative of dysfunctional neuroplasticity within discrete cortical regions.

5.1 DETERMINING A TIMELINE OF TDP-43-MEDIATED SYNAPTIC DYSFUNCTION

The first experimental chapter of this thesis sought to investigate whether the synapse is dysfunctional over a disease-time course in the *Thy1-YFP::prpTDP-43^{A315T}* (*TDP-43^{A315T}*) mouse model of ALS. It

was hypothesised that dysfunction at the postsynaptic dendritic spine is an early disease mechanism mediated by misprocessed TDP-43 and central to disease progression, due to evidence for early synaptic dysfunction in neurodegenerative disease and the normal roles for TDP-43 at the dendritic spine (Alami et al 2014, Casas et al 2016, Devlin et al 2015, Fogarty 2018, Fogarty et al 2015, Liu-Yesucevitz et al 2011, Liu-Yesucevitz et al 2014, Majumder et al 2012, Majumder et al 2016, Sunico et al 2011, Wang et al 2008a). The findings revealed reductions in dendritic spine density occurring selectively in the motor cortex at a presymptomatic time point, prior to overt cell loss, and occurring concomitantly with a reduction in the frequency of mEPSCs. This supports the notion of dendritic spine changes mediated by TDP-43 misprocessing being an early disease event in ALS, and further reveals mature spine types are specifically vulnerable to progressive alterations in dendritic spine density. The findings here evidence the tight link between the function of synapses and the regulation of dendritic spines early in disease. Mature spine types form the strongest synaptic connections, and feature large spine heads to encompass greater postsynaptic densities (PSD) (Dosemeci et al 2016, Rochefort & Konnerth 2012). The failure of postsynaptic receptors and machinery to be anchored at the PSD may potentially result in a reduced ability to strengthen synapses, and indeed TDP-43 has been linked to dendritic spine maturation through the Rac1 pathway (Majumder et al 2012). The specific dysfunction of mature spines mediated by TDP-43 misprocessing may be a result of an inability of motor cortical neurons to process activity, and therefore to strengthen synaptic connections.

Dendritic spines are essential for neuroplasticity, and regulate synaptic transmission through excitatory receptors; and indeed, it is well established that altered excitability within the motor cortex is an early pathological event in ALS (Burrell et al 2011, Menon et al 2017, Menon et al 2015, Vucic et al 2008). Here, the presymptomatic reduction in firing frequency further evidences the role of altered excitability mediating disease progression, in conjunction with dendritic spine alterations. The normal functions of TDP-43 shed light on the mechanisms that may underlie these functional and structural changes, whereby TDP-43 has been associated with the translation and regulation of AMPA receptor subunits GluA1 and GluA2 (Majumder et al 2016, Yamashita & Kwak 2014b). Accordingly, disruptions to the surface expression of these subunits results in decreased mEPSC frequency and synaptic silencing, and directly indicates TDP-43 misprocessing may be mediating the disrupted ability to respond to excitation (Liao et al 2001, Noel et al 1999). It is intriguing that the observed reduction frequency reflects hypoexcitability, as previous research has more commonly identified hyperexcitability in ALS. However, hypoexcitability has been increasingly linked to neuronal death and has been observed in iPSCs harbouring various ALS-linked mutations, including those of TDP-43 (Devlin et al 2015). Together, the findings from this chapter indicate that the complex regulation of the synaptic

compartment is altered presymptomatically in the presence of misprocessed TDP-43, resulting in dysfunction at the dendritic spine within the motor cortex. Research has yet to identify the link between TDP-43 misprocessing at the synaptic structure and the susceptibility of the motor cortex to disease insult; thus it is integral to identify fundamental features of the motor cortices that may render susceptible to pathology at the dendritic spine.

5.2 ESTABLISHING FUNDAMENTAL CHANGES TO DENDRITIC SPINES

The susceptibility of discrete neuronal networks is a key feature of neurodegenerative disease; however, the processes that render these regions vulnerable have yet to be fully elucidated. Furthermore, the influence of factors such as age and sex on dendritic spine activity within cortical regions is still poorly understood. Thus, the second experimental chapter of this thesis aimed to determine whether cortical region, age and sex influence dendritic spine changes in the *Thy1-YFP* mouse model, to establish fundamental dendritic spine activity that may render the motor cortex more exposed to activity-dependent insult. It was hypothesised that dendritic spine turnover and morphological subsets have a unique pattern of dynamics depending on cortical region, sex and age. To quantify changes at the dendritic spine, cranial window surgeries were undertaken in conjunction with 2PLSM, to capture snapshots of real-time dendritic spine turnover as modulated by age, sex and region. Spine populations were termed stable if present during all imaging rounds, or dynamic if gained, lost or matured between imaging rounds. The gain, loss or morphological maturation of a dendritic spine was considered a dynamic turnover event. Capturing the real-time changes at the dendritic spine build upon approaches classically measuring density, as the motility of the structure is essential for neuroplasticity. The findings highlight the dynamic nature of the motor cortex, and identify the region as having higher activity demands in comparison to the somatosensory cortex. Additionally, the role for cycling oestrogen in regulating dendritic spine changes underlying regional neuroplasticity is key to understanding the sexual-dimorphism of cortical development and regulation both in health and in disease.

The shaping of cortical circuits is dependent on dendritic spine reductions, preceded by peaks in dendritic spine density that indicate a greater capacity for neuronal plasticity during critical periods (Arikkath 2012, Boivin et al 2018, Bourne & Harris 2008, Flurkey et al 2007, Gonzalez-Burgos et al 2015, Johnson et al 2016, Knott & Holtmaat 2008, Koss et al 2014, Petanjek et al 2011, Poon 2018, Selemon 2013). The P60 time point explored in this Chapter represents a period of potentially increased plasticity, as evidenced by an increase in dendritic spine density preceding overall spine reductions by P90. Cortical regions undergo differential timelines of circuit development whereby

higher order regions refine synaptic connections later than lower order regions. The motor cortex is a region that consists of vast cortical networks interconnected to frontal and subcortical regions, with evidence for high synaptic demands, particularly during periods of heightened plasticity (Barthas & Kwan 2017, Brecht 2011, Kolb et al 2012, Lein et al 2006, Meunier et al 2009). The increase in mature spine turnover observed here may indeed reflect this, whereby mature spines within the motor cortex require greater synaptic inputs to be strengthened (Taft & Turrgiano 2013; Lambert et al 2017).The decrease in immature dendritic spine turnover by adulthood further highlights the maturation of the motor cortex, where maintained mature dendritic spine dynamics may be required to regulate the dense connectivity of the motor region.

Perhaps one of the more intriguing results from the current Chapter is that of how cycling oestrogen regulates dendritic spine populations within different cortical regions. Baseline levels of oestrogen in the motor cortex are associated with high dynamic dendritic spine populations, whilst transient peaks of high oestrogen are associated with stable dendritic spine populations. Increases in spine density have been associated with increased oestrogen levels, and this is thought to be an initiating step in forming and stabilising dendritic spines; thus potentially, cycling oestrogen peaks may be initiating crucial stabilisation events underlying neuroplasticity (Alexander et al 2018; Wang et al 2018). Furthermore, oestrogen within the motor cortices appears to influence mature spine types, with these stronger connections associated with greater AMPA receptor recruitment at the dendritic spine head (Babayan & Kramár 2013, Kramár et al 2009, Lee et al 2003, Woolley & McEwen 1994). Studies have identified that the targeted stimulation of oestrogen receptors is linked to greater recruitment of GluA1 and GluA2, and it may be that findings in the current thesis are underpinned by the transient stabilisation of dendritic spines within the motor cortex by the recruitment of AMPA subunits.

The somatosensory cortex displays differential trajectories between the sexes for the balance of dynamic and stable dendritic spine populations over time, and this may further reflect the oestrogen-mediated timelines for cortical development (Qiu et al 2018). The evidence here suggests the female somatosensory cortex may mature earlier than that of males; maintained dynamic proportions of dendritic spines in females may indeed contribute to the strengthening of synaptic connections over time, maintaining neuroplasticity. While steady state conditions in the female somatosensory cortex have been shown to be unaffected by oestrus cycling, the ability of neurons in this region to respond to activity is influenced by oestrogen levels, whereby once more high oestrus is associated with higher dendritic spine turnover (Alexander et al 2018). These findings shed further light on the activity profile that may define the mature female somatosensory cortex, highlighting that maintained dynamic

dendritic spine populations play a role in regional networks through the regulation of synaptic connections.

Collectively, this Chapter identifies the key characteristics of two distinct cortical regions that confer vulnerability to one – the motor cortex – in disease states, whilst the other – the somatosensory cortex- is spared during the earlier stages of disease progression. The high dynamics of the motor cortex suggest the region may be more susceptible to activity-dependent insults, with cycling oestrogen playing a critical role in the transient stabilisation of neuroplastic mechanisms. The findings here further highlight the distinct differences between lower and higher order cortical circuitry over time, whereby lower order regions display distinct timelines of maturation between the sexes. Whilst shedding light on some of the normal changes occuring at the dendritic spine, this Chapter also raises many questions as to how dendritic spines respond to the presence of a disease linked protein mutation within a vulnerable cortical region.

5.3 IDENTIFYING EARLY ALTERATIONS TO NEUROPLASTICITY AT THE DENDRITIC SPINE IN DISEASE

The results from the first two experimental chapters set the stage for the final study of this thesis, observing early synaptic dysfunction within the motor cortex at the dendritic spine that is linked to perturbations in fundamental neuroplasticity of the region. Furthermore, the differential stable and dynamic dendritic spine proportions between males and females experiencing high oestrogen indicate a key role for oestrogen in the response of dendritic spines to activity in disease states.

The male *TDP-43^{A315T}* motor cortices display a reduced dynamic proportion of dendritic spines, mirrored by the overall reduction of mature spine turnover. The motor cortex is an innately active region, being a higher order cortical region and featuring local, intercortical and subcortical networks associated with classical motor and cognitive functions (Auer et al 2018, Duncan & Owen 2000, Sanes & Donoghue 1997). For the motor cortex to function appropriately, it requires the rapid establishment of modifiable synaptic connections – this need for heightened neuroplasticity may indeed be what induces activity-dependent susceptibility in the presence of a TDP-43 mutation (Chen et al 1998, Ziemann et al 1998). The time point at which susceptibility at the dendritic spine has been identified in the current thesis is normally one of increased spine density overall, and should be indicative of a vast capacity for neuroplasticity. However, it is during these periods that regions such as the motor cortex are most likely to sustain insult from high activity demands in the presence of protein misprocessing (Purves et al 1988, Schaefers & Teuchert-Noodt 2016). The evidence here suggests that

in TDP-43-mediated disease states, perturbations of the dendritic spine may be contributing to disease progression during a preclinical period, particularly in males.

Conversely, female *TDP-43^{A315T}* mice display increased dynamics of dendritic spines within the motor cortex regardless of oestrogen level. Intriguingly, this is in comparison to normal conditions of cycling oestrogen in the female motor cortex whereby high oestrus is associated with stable dendritic spine populations. Oestrogen has well-documented neuroprotective effects, and the increased dynamics observed in the *TDP-43^{A315T}* motor cortex may be attributed to compensatory mechanisms aimed at maintaining high dynamics in a highly active cortical region. The production of oestrogen at the synapse has been directly linked to the stimulation of synapses, and further ties activity-dependent mechanisms to functions at the dendritic spine (Zhou et al 2014). Though this provides a potential pathway through which to rescue dendritic spine dysfunction, it is important to note that work from others demonstrates the need for incredibly targeted applications of oestrogen for neuroprotection; if used too late in disease it is ineffective, and it is still unknown which specific oestrogen receptors need to be stimulated at what time to exert neuroprotective effects (Brinton 2008, Nilsen 2008, Scott et al 2012). To effectively target early disease events and potentially halt pathological progression, it is crucial to identify the optimal timing of an oestrogen agonist that stimulates the correct receptors in order to maintain fundamental neuroplasticity at the dendritic spine.

The first experimental chapter identified specific alterations to mature spines, shown to be integral to fundamental neuroplasticity within the motor cortex in both males and females in Chapter 3. In the final chapter, *TDP-43^{A315T}* females display increases in the gain of mature spines during high oestrogen levels, whilst during baseline oestrogen levels there is instead a reduction in the overall gain of these spine types. TDP-43 has been implicated in roles in dendritic spine maturation through the negative regulation of the Rac1 pathway and the translation of the AMPA subunit GluA1 (Majumder et al 2012; Majumder et al 2016). With normal roles in dendritic spine formation and maturity, misprocessed TDP-43 may be responsible for dysfunction at this specific spine type in disease states. With roles for TDP-43 in trafficking mRNA and RNA to the synapse, dysfunction may result in an inability of AMPA receptor subunit mRNA to reach the dendritic spine. A reduced ability to traffic key synaptic proteins to the dendritic spine for localised protein synthesis may then result in an inability to respond to activity. Thus potential protective effects observed during high oestrogen in the *TDP-43^{A315T}* female may be concentrated on preserving the dynamic turnover of mature dendritic spines specifically, in order to maintain strengthened cortical circuits in a highly active region.

5.4 FUTURE DIRECTIONS AND LIMITATIONS

This thesis focused on characterising early disease events mediated by TDP-43 at the synapse, establishing fundamental changes at the dendritic spine underlying neuroplasticity. The high activity demands of the region may render the motor cortex susceptible to pathology, and the potential for cycling oestrogen to protect against dendritic spine dysfunction in the *TDP-43^{A315T}* mouse model of ALS further reveals a mechanism that may explain sexual dimorphism in neurodegenerative diseases. For these findings to be translated into new pathways for viable therapeutics aimed at targeting preclinical periods of disease, it is crucial to undertake further studies to understand the mechanisms underlying the changes observed at the dendritic spine.

The use of the *Thy1-YFP* transgene enables high-resolution visualisation of dendritic spines within control and pathological conditions. However, as YFP preferentially labels a subset of motor and sensory neuronal populations, the reduction of YFP-positive neurons as observed may not correspond to overall cell death. To identify the scale of neurodegeneration as a consequence of TDP-43 misprocessing, further studies may utilise NeuN or Tunel staining in order to quantify total neuronal loss. Further, while the measures of dendritic spine density and turnover provide a read-out of changes occurring at the dendritic spine and the availability of certain synaptic connections, these cannot account for the potentially variable viability of synaptic machinery at dendritic spines both normally and in disease; nor can they account for the molecular mechanisms underlying dendritic spine changes. To further investigate the processes underlying modifications to dendritic spines, key synaptic proteins at the synaptic membrane need to be quantified. The composition of AMPA and NMDA receptor subunits at the postsynaptic membrane regulates the function of the dendritic spine, and with TDP-43 linked particularly to AMPA processing, probing receptor proteins may provide answers as to how TDP-43 mediates synaptic alterations. Experimental paradigms that may be utilised to achieve this include targeted PCR arrays and identifying splicing targets of TDP-43 in the *TDP-43^{A315T}* model of ALS. Performing PCR arrays on synaptosomes taken from the mouse model will allow for optimised protocols to identify receptor subunits or postsynaptic machinery that may be altered in the presence of protein misprocessing. Additionally, exon-intron-specific RT-PCR may be used to further determine proteins and subunits that undergo alternative slicing in disease states within the PSD. TDP-43 regulates the alternative slicing of a range of RNAs, yet studies have not targeted this approach to specific neuronal targets (Deshaies et al 2018, Tollervey et al 2011). By expanding such an approach to measure splice targets within whole-cell samples, synaptosomes and subproteomes of the PSD, the differential regulation of synaptic proteins within cellular compartments may be used to elucidate where TDP-43 may be exerting toxic effects on key synaptic molecules.

The specific alterations to mature spine types in the current thesis - along with evidence for overall changes in neuroplasticity mediated by TDP-43 misprocessing – are indicative of the essential role of mature spines as strengthened synaptic compartments. As aforementioned, AMPA subunits are critical for the maturation of dendritic spines, and regulate processes underlying activity-dependent neuroplasticity through continual receptor insertion, anchoring and diffusion events (Anggono & Huganir 2012). To establish the potential for AMPA receptor function to be perturbed at specific subunits, insertion events may be driven using an optical dimerization system (Kennedy et al 2010). This system can be used to locally control the rapid recruitment of AMPA receptors to the PSD following activity (Kennedy et al 2010, Sinnen et al 2017). These experiments can then be used in conjunction with electrophysiological measurements of mEPSCs, in order to determine whether the optogenetic insertion of AMPA subunits such as GluA1 may rescue the hypoexcitable phenotype observed in the current thesis. Undertaking this experimental paradigm within layer V pyramidal neurons of the motor cortices and other cortical regions may further highlight the mechanisms underlying the vulnerability of the motor cortex in the presence of TDP-43 misprocessing.

The electrophysiological experiments in Chapter 2 were carried out at P30 and P60, with a hypoexcitable phenotype identified at P60 and the changes identified in dendritic spine changes in the *TDP-43^{A315T}* motor cortex further indicative of presymptomatic hypoexcitability. Interestingly, within the literature hyperexcitability has been most frequently associated with early ALS pathology (Bae et al 2013, Menon et al 2017). To account for these apparently contrasting findings, the current thesis may be capturing a specific snapshot of altered excitability in a network that no longer has the capacity to regulate activity-dependent neuroplasticity, with excitotoxicity arising from both hyper- and hypo-mechanisms over time. Indeed, within ALS-patient derived iPSCs, fluctuations in excitability are evident with a switch from hyperexcitability to eventual hypoexcitability (Devlin et al 2015, Leroy & Zytnicki 2015). To establish a timeline of excitability changes in the *TDP-43^{A315T}* cortex, it is essential to quantify the frequency and amplitude of mEPSCs further over the time course, in order to identify how the susceptible motor cortex may be attempting to compensate for the lost ability to modulate networks.

The ability to respond to stimulation and modulate synaptic connections following activity underpins cortical neuroplasticity; critical for learning and memory. Though the changes identified in the current thesis at the dendritic spine are indicative of altered neuroplasticity, it is still unknown as to whether this then impacts the ability to create new synaptic circuits within discrete cortical regions. For future studies to capture this process, it would be beneficial to undertake the 2PLSM paradigm of the current thesis and use this in conjunction with targeted behavioural experiments. Ensuring these paradigms

are optimised to stimulate discrete cortical regions will facilitate further identifying processes underlying select vulnerability in disease, and highlight the potential for TDP-43 misprocessing to be impacting the maturation and strengthening of synaptic connections during activity. To stimulate the motor and somatosensory cortices, rotarod and tactile experiments may be utilised respectively in male and female controls and in *TDP-43^{A315T}* mice, uncovering both the impact of mutated protein and the oestrus cycle on the ability to create new synaptic connections within discrete cortical regions.

The findings in Chapters 3 and 4 regarding the role of cycling oestrogen – particularly in the motor cortex – uncover a range of questions as to how oestrogen may be mediating dendritic spine dynamics and stabilisation, as well as how it may exert neuroprotective effects. To further utilise 2PLSM experiments, another consideration would be to utilise female mice that have undergone an ovariectomy, in order to identify whether the loss of oestrogen results in a loss of dendritic spine dynamics and synaptic dysfunction. The RT-PCR experiments detailed above may build upon this by identifying synaptic compartment-specific changes in distinct oestrogen receptors in the presence of *TDP-43^{A315T}*; agonists of altered receptors may then be used to potentially restore dendritic spine dynamics reduced in females that have undergone ovariectomies.

A number of studies suggest anesthesia commonly used during surgery – such as Ketamine – may alter the plasticity of dendritic spines, though findings vary. While the systemic use of Ketamine is associated with the increased dendritic spine density of layer V pyramidal neurons in a range of cortical regions in rats, hippocampal neurons *in vitro* exposed to chronic Ketamine demonstrate significant reductions in both dendritic spine density and length (Jiang et al 2018, Phoumthipphavong et al 2016, Li et al 2011, Li et al 2010). Intriguingly, region-specific alterations at the dendritic spine have been identified following ketamine application, whereby acute, low doses increase dendritic spine density of layer V pyramidal neurons in the medial frontal cortex with no change in the striatum (Ruddy et al 2015, Li et al 2011, Li et al 2010). However the majority of these findings are in either juvenile or aged mice; indeed, anesthetic use of Ketamine in conjunction with Xylazine has been shown to have only transient effects on filopodia in 1 month old mice, with no lasting changes at the dendritic spine (Yang et al 2011). Isoflurane, another commonly used anesthesia for surgery, has also been shown to impact dendritic spines, particularly at juvenile stages (Yang et al 2011, Briner et al 2010, Head et al 2009). Though the impacts of anesthesia on the dendritic spine have the potential to present a confounding variable, ensuring the parameters of anesthetic use are maintained across all experimental groups is crucial for comparable results. The use of Ketamine/Xylazine in the current study for 2PLSM was standardized for all experimental groups, with the timepoints employed in Chapter's 3 and 4 beyond the age considered most susceptible to alterations associated with Ketamine application.

As with many experiments within a specific mutant mouse model of disease, it is essential to undertake experimental paradigms in other models of ALS and TDP-43 misprocessing, to understanding whether the changes identified are representative of a specific TDP-43 mutation or are a general consequence of TDP-43 dysregulation. The use of other TDP-43 mutants – such as the M337V or Q331K mutations –may provide a means to answer this question, and indeed studies utilising varying mutations of TDP-43 have also identified early changes to dendritic spines (Fogarty et al 2016, Fogarty et al 2015). Furthermore, the use of mouse models featuring inducible TDP-43 mislocalisation in targeted cortical regions may enable the identification of dysfunction that is directly linked to protein aggregation, and further elucidate whether this pathology is linked to familial disease forms, or is a wider consequence of TDP-43 misprocessing (Spiller et al 2016, Walker et al 2015). The aggregation of TDP-43 is a hallmark feature of ALS, and the ability to associate protein aggregation with changes at the dendritic spine may provide critical clues as to how sporadic ALS pathology may progress. The use of mouse models featuring inducible TDP-43 mislocalisation in targeted cortical regions may enable the identification of dysfunction that is directly linked to protein aggregation, and further elucidate whether this pathology is linked to familial disease forms, or is a wider consequence of TDP-43 misprocessing.

As a whole, this thesis has highlighted the changes at the dendritic spine that may be underlying the sexually-dimorphic vulnerability of the motor cortex to TDP-43-mediated forms of ALS. The findings here also emphasise the critical role of age and sex on the differential development of cortical regions, with implications for the susceptibility of neuronal populations to neurodegenerative disease. Changes in neuroplasticity at the dendritic spine have been identified at a presymptomatic time point in the *TDP-43^{A315T}* mouse model of ALS, highlighting the need to understand early disease events in order to produce viable therapeutic targets aimed at slowing or halting disease progression at the synapse. Future studies are required to elucidate the molecular mechanisms underlying these changes, as well as the effects this then has on the ability to form new synaptic connections. Undertaking these experiments in a range of models of disease, as well as in female mice lacking oestrogen, may shed further light on the processes mediating altered neuroplasticity of the motor cortex in the presence of dysregulated TDP-43 at the dendritic spine.

5.5 CONCLUSIONS

The select vulnerability of cortical regions to neurodegenerative mechanisms is thought to be a product of pathological pathways active well before the clinical manifestation of disease. Synaptic dysfunction has been documented as occurring within susceptible regions during early disease stages, and in ALS early alterations to excitability may be intrinsically linked to activity at the synapse. This thesis provides significant evidence that altered neuroplasticity at the dendritic spine plays an early role in the progression of ALS in the *TDP-43^{A315T}* mouse model, and that TDP-43 itself has key roles in the regulation of dendritic spine changes. It also furthers knowledge of the neuroplastic mechanisms underlying sexual-dimorphism in both the normal development of synaptic networks within discrete cortical regions, as well as how the cycling of the sex hormone oestrogen exerts neuroprotective maintenance of these networks in disease states. The findings indicate the male motor cortex is specifically vulnerable to TDP-43-mediated dysregulation of neuroplasticity at the dendritic spine, occurring early in the disease course within a cortical region primed to fail through activity-dependent insults. Understanding the factors influencing the select susceptibility of cortical regions and neuronal populations to disease is crucial for targeting early pathological events. Identifying oestrogen as a key regulator of neuroplasticity and a potential neuroprotective target provides a therapeutic avenue for future studies to optimise the application of oestrogen agonists to restore functions at the dendritic spine in ALS. Developing treatments that can successfully target early disease mechanisms is crucial to halting or slowing the progression of this devastating disease.

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